Effect of non-pathogenic bacteria on plant pathogen virulence and the plant immune system

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Abstract

The development of so-called biocontrol agents is in the spotlight to solve agricultural losses caused by foliar pathogens. Biocontrol agents can protect plants via direct antagonistic interactions or by modulating host immune networks to induce plant resistance indirectly. The focus of this thesis lies on screening potential microbial biocontrol agents against model pathogens and dissecting the underlying mechanisms behind such protection.

I have employed an *in planta* assay to inoculate gnotobiotic Arabidopsis with individual strains from a diverse set of bacteria prior to infection with either the model biotrophic pathogen *Pseudomonas syringae* DC3000 or the model necrotrophic pathogen *Botrytis cinerea*. The protective ability of each bacterial strain was determined by examining the plant phenotype. The direct antagonistic interactions were suggested on a bacterial population level. In addition, a protoplast-based assay was established to investigate the potential link between the reduction in disease phenotype and the potential modulation of the plant immune responses to bacteria.

As a result, *Acidovorax* sp. leaf 84, *Pedobacter* sp. leaf 194, *Plantibacter* sp. leaf 1 and *Pseudomonas citronellolis* P3B5 in addition to *Sphingomonas* species showed a striking ability to protect plants from developing severe disease symptoms caused by biotrophic pathogen *Pseudomonas syringae* DC3000 infection. *Arthrobacter* sp. leaf 145, *Pseudomonas syringae* DC3000, *Pseudomonas syringa*e B728a, *Pantoea vagans* PW, *Pantoea vagans* C9-1, *Pantoea agglomerans* 299R, *Rhodococcus* sp. leaf 225, *Sphingomonas* sp. leaf 17, *Sphingomonas phyllosphaerae* FA2 and *Sphingomonas melonis* FR1 showed plant-protection in reducing plant mortality rate infected by necrotrophic pathogen *Botrytis cinerea*. Interestingly, the mode of *Pseudomonas syringae* DC3000 induced disease suppression seems to be different based on the bacterial strains used: *A.* sp. leaf 84 and *P. citronellolis* P3B5 significantly reduced the *Pseudomonas syringae* DC3000 population size *in planta* while *P. citronellolis* P3B5, *P. syringa*e B728a, *P. vagans* C9-1, *P. agglomerans* 299R, *S.* sp. leaf 17 and *S. phyllosphaerae* FA2 induced a strong plant immune response in the protoplast assay. This suggests *A.* sp. leaf 84 may only depend on direct antagonistic interactions to diminish pathogen proliferation while the other protective strains can employ plant immune systems to confer their protection. Moreover, a

few protective strains such as *P. syringa*e B728a against Botrytis induced salicylic acid signalling responses in the protoplast assay, which infers the positive effect of salicylic acid signalling on defence against necrotrophic pathogens. This study has successfully screened the biocontrol agents against model pathogens and furthers our understanding of biocontrol activity, thereby aiding to achieve improved disease control.

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Abbreviation

Abbreviations used in this thesis:

Arbitrary fluorescence units	au
Colony forming units	cfu
Days post inoculation	dpi
Effector-triggered immunity	ETI
Hypersensitive response	HR
Jasmonic acid	JA
Microbe-associated molecular patterns	MAMPs
Pathogenesis-Related Gene 1	*PR1*
Pattern-triggered immunity	PTI
Pattern-recognition receptors	PRRs
***Pseudomonas syringae* pv. *tomato* DC3000**	Pst
Salicylic acid	SA
Type III secretion systems	T3SS
Vegetative Storage Protein 1	*VSP1*

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Chapter 1. Literature review and research questions

1.1 Microbial ecology of bacteria on leaves

Leaves, as an important part of the above-ground surface of plants (phyllosphere), host a considerable number of microorganisms, mostly bacteria (Schlechter et al., 2019). Among these microorganisms, some have beneficial or no effect on the plant host, these are so-called non-pathogens, whereas others with a negative effect are called pathogens. Pathogens can be divided into two classes, biotrophic and necrotrophic pathogens (Glazebrook, 2005). Biotrophs feed on living plant tissues, while necrotrophs kill host tissues and feed on the remains.

In addition to these fundamental effects of microbes on plant hosts, there are various interactions between microbes. Cooperation, direct antagonism and indirect antagonism are the three common microbe-microbe interactions (Glazebrook, 2005; Koskella & Brockhurst, 2014). Cooperation describes relationships where at least one partner gains benefits from the interaction, while the other is not impacted (Schlechter et al., 2019). Direct antagonisms involve direct inhibition of other organisms by toxic secretions or direct injection of antibiotics or hydrolytic enzymes (Boller, 1993; Chin-A-Woeng et al., 2003). During indirect antagonism one species inhibits the growth of another indirectly, normally by resource competition such as nutrients or space (Leveau & Lindow, 2001; Remus-Emsermann, Kowalchuk, et al., 2013; Zeriouh et al., 2014). With regard to competition for nutrients and resources, microbes have evolved different strategies such as improving their mobility, resource binding and uptake efficiency (Vorholt, 2012; Wensing et al., 2010). Another form of indirect antagonism is the production of surfactins by *Bacillus subtilis.* Surfactin production advances the formation of biofilms in *Bacillus subtilis* and thereby provides a competitive advantage on its ability to colonise plants (Wei et al., 2016). Interestingly, surfactins also have an antibacterial activity against the plant pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (Pst) (Bais et al., 2004). Yet another form of indirect antagonism is the interference with microbial communication by degrading quorum sensing molecules (Alymanesh et al., 2016).

Recent evidence suggests that the majority of microbial interactions are negative interactions such as competitive relationships. The presence of bacteria and their negative interactions with eukaryotic root microbes can protect the plant host from disease and even

promote plant growth (Durán et al., 2018). Innerebner et al. (2011) have shown that the pre-colonisation of leaves with some species of the genus *Sphingomonas* protects plants from pathogen attack. This was evident through a correlation in the reduction of pathogen numbers. The causal reason for the interaction is unclear, but it has been suggested that plant-protective effect might be a result from direct microbe-microbe interactions. Furthermore, there is increasing evidence that not only microbe-microbe interactions are responsible for plant protection, but also plant host responses to non-pathogenic colonisers may affect pathogen colonisation and disease (Vogel et al., 2016). In other words, the whole relationship works as tritagonism (Freimoser et al., 2016), in which the plant host plays a role as a mediator between bacterial colonisers. How plants mediate microbial colonisation will be introduced in the next section.

1.2 A brief history of the plant innate immune system

In 1905, Biffen first described the inheritance of pathogen-resistance in wheat (Biffen, 1905). In the 1940s, Flor (1971) proposed the hypothesis of "gene for gene" resistance, which laid the foundation for the study of plant immunology. By cloning and characterising the plant disease resistance genes, it has been found that the immune receptors encoded by plant disease resistance genes are similar to the innate immune receptors found in fruit flies and mammals in the 1990s (Wood, 2004). It was recognised that plants do not have adaptive immunity like vertebrates, but they have innate immune mechanisms similar to animals (Wood, 2004). The current framework of plant immunity was formulated 100 years after the discovery that immunity is based on inheritable genes. This framework states that in order to recognise pests and pathogens, plants use two different tiers of immune receptors located on the plasma membrane and in intracellular spaces (Chisholm et al., 2006; Jones & Dangl, 2006).

The first tier is pattern-triggered immunity (PTI), in which plants recognise microbe-associated molecular patterns (MAMPs) by pattern-recognition receptors (PRRs) which comprise receptor-like kinases and receptor-like proteins (Bigeard et al., 2015a; Mukhtar et al., 2011a). In most cases, MAMPs are conserved molecules shared by a wide range of microbes, which are essential for microbial survival and fitness (Boller & Felix, 2009). In the past two decades, numerous MAMPs such as proteins (e.g. flagellin), elongation factor thermo unstable, lipopolysaccharide, and carbohydrates (e.g. chitin, elicitin) have been characterised together with their cognate PRRs (Boutrot & Zipfel, 2017). Upon MAMP binding, these immune receptors activate the plant immune system. In order to interfere with

the immune response of a plant host cell, plant pathogens will release effectors into the environment to facilitate their infection or colonisation. Effectors are pathogen-synthesised molecules delivered into plant cells to overcome PTI and interfere with the host immune system (Bigeard et al., 2015; Mukhtar et al., 2011). To counteract this interference, plants developed a second tier of immunity. Briefly, the second tier of plant immunity is called effector-triggered immunity (ETI) in which plants use disease resistance proteins to recognise pathogen effectors. Plant Resistance genes often encode nucleotide binding-leucine-rich repeat receptors that recognise pathogen effectors (Schlechter et al., 2019; L. Wu et al., 2014).

Phytohormone signalling networks play important roles in PTI and ETI (Tsuda et al., 2009). The two most well-characterised plant hormones salicylic acid (SA) and jasmonic acid (JA) play a key role in plant immunity as they act against biotrophs and necrotrophs, respectively (Glazebrook, 2005) (Fig 1.1). For example, SA production levels reach a peak at 9 h after flagellin treatment (Tsuda, Sato, et al., 2008). JA is produced within 24 h after treatment with the Pep-13 MAMP (Liu & Zhang, 2004; Tsuda, Sato, et al., 2008). In accordance with their respective responsibility, SA and JA signalling pathways have been shown to antagonise each other (Bostock, 2005; Glazebrook, 2005; Vlot et al., 2009). Moreover, a number of studies have shown that SA signalling plays a dominant role in this antagonistic relationship, i.e. that more JA-related genes are repressed by SA signalling and that the SA pathway has a higher priority in terms of gene regulation (Glazebrook et al., 2003; Koornneef et al., 2008). During ETI, SA regulates the hypersensitive response (HR) which results in the formation of concentric cell death around the site of infection to cut biotrophic pathogens off from key resources (Betsuyaku et al., 2018). JA is activated simultaneously around the site of HR, likely to restrict propagation of the SA signal into uninfected areas (Betsuyaku et al., 2018). In addition to the recognition system based on immune receptors, plants also have a gene silencing system mediated by microRNA, which can recognise and defend the invasion of viruses and other pathogens (Zvereva & Pooggin, 2012).

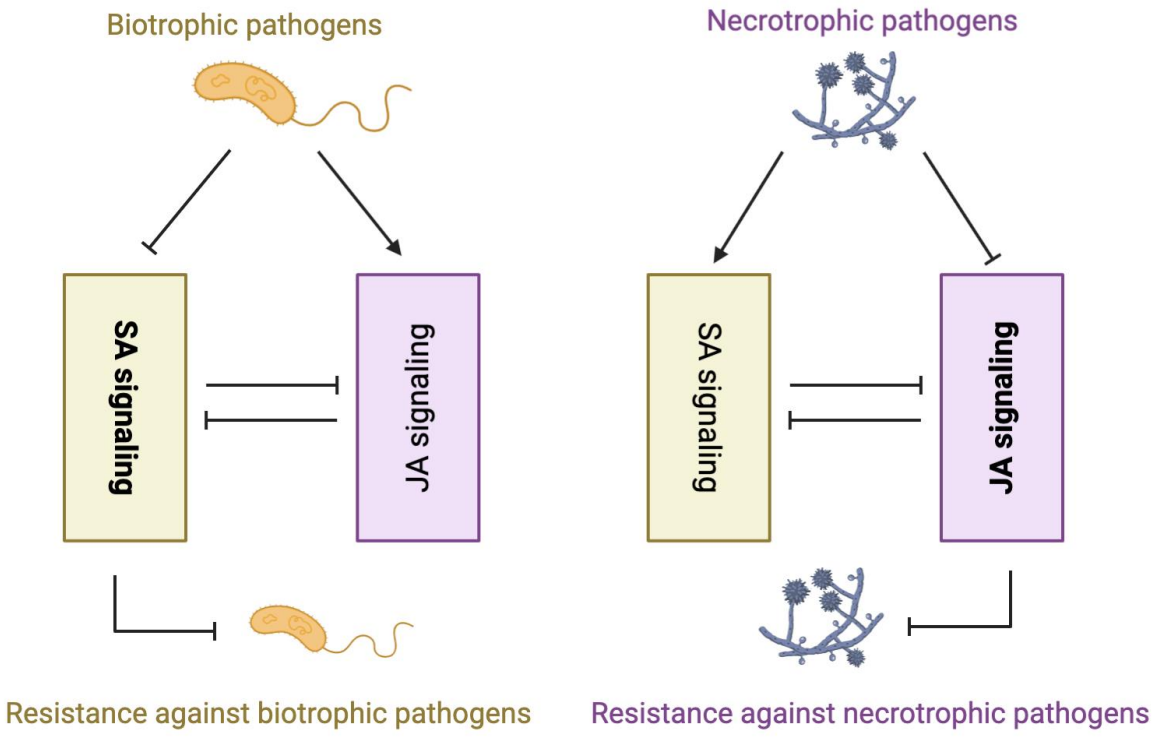


Figure 1. 1 The role of SA and JA signalling pathways in the defence of plants against pathogens. Biotrophic pathogens suppress SA signalling while activating JA signalling. By contrast, necrotrophic pathogens suppress JA signalling but activate SA signalling. SA and JA signalling dependent immunity are responsible for resistance against biotrophic pathogens and necrotrophic pathogens, respectively. Meanwhile SA and JA have an antagonistic relationship.

1.3 The effect of plant immune system on pathogen virulence

Although plants have set a range of barriers to protect themselves from pathogens, bacteria and fungi also have evolved different strategies to breach these barriers. Fungi use their hyphae to penetrate the leaf epidermis physically or invade stomata (Zeng et al., 2010). By contrast, bacterial pathogens invade into leaf tissues via natural openings such as stomates or hydathodes, or via wounds. Their colonisation success can depend on the overall perturbation of the plant immune system (Han, 2019; Vogel et al., 2016). Since plant hosts use phytohormone signalling to orchestrate immune responses, one strategy of pathogens is to produce phytohormone mimics to interfere with hormone signalling pathways. The model biotrophic pathogen, *Pseudomonas syringae* pv. *tomato* DC3000 (Pst), produces a mimic phytotoxin (coronatine) of JA-Ile, the active form of JA, to up-regulate the JA signalling pathway and increase the susceptibility of their plant hosts (Katsir et al., 2008). This strategy has been corroborated by JA insensitive Arabidopsis mutants which have higher resistance to many biotrophic microorganisms such as *P. syringae and Fusarium oxysporum* (Anderson

et al., 2004; Fernández-Calvo et al., 2011). Furthermore, evidence provided by Vogel et al. (2016) showed that genes related to JA response were significantly upregulated in Arabidopsis after Pst infection. Similarly, the expression of JA-dependent defence genes will be induced to trigger plant resistance to *B. cinerea* infection (An & Mou, 2013). In addition, *B. cinerea* could induce SA signalling by producing an exopolysaccharide to antagonise JA signalling and therefore enhance its pathogenesis in tomato (Taha, 2011). Type III secretion systems (T3SS) are another important tool evolved by bacterial pathogens to perturbate the plant immune response. Various virulence-associated proteins translocated by T3SS can be harnessed by pathogens to overcome PTI, the first layer of the plant immune system (L. Wu et al., 2014). These so-called effector proteins or for short effectors are directly injected into plant cells (Büttner, 2016) and may interfere with hormone signalling pathways. For example, *P. syringae* produces the effectors HopD1 and HopI1 to reduce SA levels (Büttner, 2016). *Xanthomonas. campestris pv. vesicatoria* secretes the bacterial toxin syringolin and the effector XopJ to suppress SA signalling (Robert-Seilaniantz et al., 2011). Another form of effectors derived from *B. cinerea* are small RNA (sRNA). These fungal effectors can hijack plant RNA interference pathways and suppress plant immunity (Weiberg et al., 2013).

1.4 Rationale and research summary

Currently there are more than 40 countries that have issues with food shortage and almost 800 million people do not have adequate food for supporting basic healthy life (Food and Agriculture Organization of the United Nations, n.d.). Plant diseases including crop yield reduction and quality decline caused by pathogens are main threats to agricultural production in countries around the world and cause at least 10% yearly crop yield loss (Strange & Scott, 2005). To mitigate yield loss, pesticides are extensively used. In China, for instance 561.12 million hectares are treated with pesticides per year in order to control agricultural diseases and pests, which is 4.16 times of the country's arable land area, that is, the average prevention and control of each arable land is carried out at least 4 times a year (Zhou et al., 2019). Using pesticides causes tremendous pressure on the environment and human health alike (Jepson et al., 2020). Therefore, research on environmentally friendly disease and pest control strategies is crucially important.

Developing biocontrol agents or pathogen-resistant plant varieties are the two main alternative strategies to control pathogens which both have advantages of being sustainable (Labuschagne et al., 2010). Profound understandings of microbe-microbe interactions and plant immunology are vital for the development of those approaches. My project intends to

further our understanding of disease control and plant-microbe interactions in the phyllosphere. Recent evidence shows that plants can be protected from pathogens such as filamentous fungi and bacteria by specific bacterial communities (Durán et al., 2018; Innerebner et al., 2011; Vogel et al., 2012). The mode of action of the biocontrol can be conferred by direct microbe-microbe interactions or indirect interactions via the plant host (Helfrich et al., 2018; Jones & Dangl, 2006). Therefore, my first hypothesis is that non-pathogenic bacteria inhibit the proliferation of the biotrophic pathogen Pst or the hyphal growth of the necrotrophic pathogen *Botrytis cinerea* and thereby protect the plant host (Fig 1.2). *In planta* assays were used to determine the macroscopic phenotype of Arabidopsis and microbial quantification. The second hypothesis is that the plant host immune response is influenced by non-pathogenic bacterial strains and that the strains play an important role in shaping pathogen Pst proliferation. In order to elucidate the roles of plant immune networks in response to different plant microbiota members, I established a gnotobiotic plant protoplast assay. This protoplast assay allows the detection of real-time plant immune marker expression in a high throughput fashion, using transcriptional reporter lines, expressing a fluorescent marker.

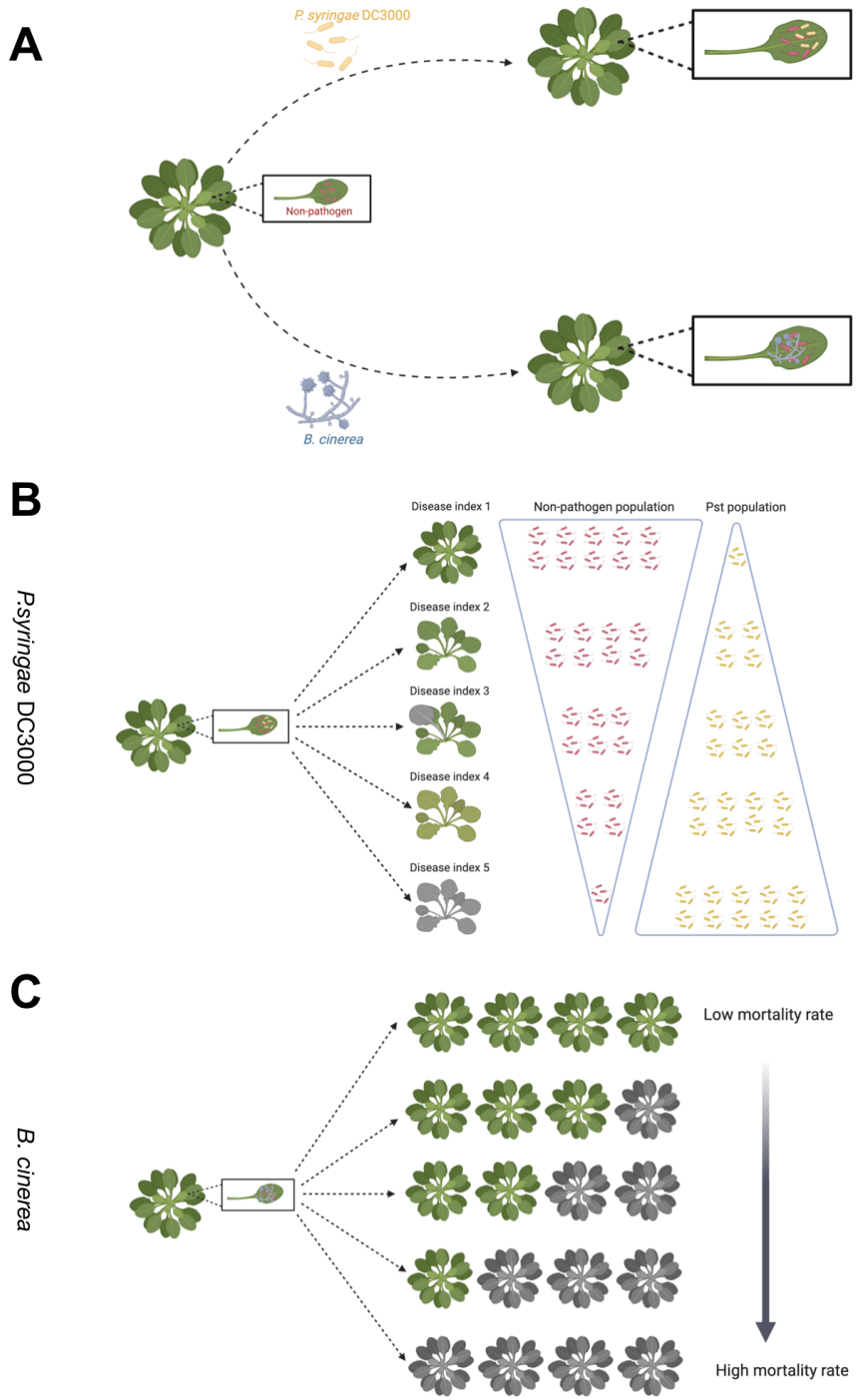


Figure 1. 2 The brief process of experimental design and expected outcomes. (A) To test the protective ability of non-pathogenic bacteria against Pst or B. cinerea, 14-day-old Arabidopsis are inoculated with individual non-pathogenic strains. Then, the pre-inoculated plants are infected with Pst or *B. cinerea*. To assess changes in infection, plant phenotypes and bacterial densities are determined. (B) Expected outcome of plants co-colonised with non-pathogenic bacteria and Pst. Plants colonised by highly protective strains will show less disease symptoms and host higher abundances of non-pathogenic bacteria and a lower abundance of Pst. Plants colonised by nonprotective strains will show more severe disease symptoms and host lower abundances of non-pathogenic bacteria and a higher abundance of Pst. (C) Expected outcome of plants co-colonised with non-pathogens and B. cinerea. Plants colonised by highly protective strains will be more resistant to *B. cinerea* infection resulting in a lower mortality rate. Plants colonised by nonprotective strains will be less resistant to *B. cinerea* infection resulting in a higher mortality rate.

Chapter 2. A plant phenotypic screen to determine plant protection of non-pathogenic bacteria against *Pseudomonas syringae* DC3000 and *Botrytis cinerea*

2.1 Introduction

It was previously reported that *Sphingomonas* spp. can protect Arabidopsis against Pst (Innerebner et al., 2011). This protection was accompanied with lower pathogen populations compared to mock inoculated plants. In addition, growth of the necrotrophic pathogen *B. cinerea* was inhibited by certain *Pseudomonas* strains (Völksch & May, 2001). This evidence shows that bacterial biocontrol agents are able to suppress plant diseases caused by bacterial and fungal pathogens (De Vrieze et al., 2018; Hunziker et al., 2015; Simionato et al., 2017). More and more research is investigating these microbial biocontrol activity on plants. It is highly desirable to determine additional bacterial groups that exhibit properties that make them effective biocontrol agents to further build on this biocontrol strategy.

To contribute to this contemporary endeavour, I screened a diverse set of non-pathogenic bacterial taxa (18 genera, Fig 2.1) in a gnotobiotic *in planta* assay. Plants were pre-colonised by individual non-pathogenic strains before pathogen infection. The plant-protective ability of these non-pathogenic bacteria was determined by rating the plant disease symptoms in case of infection with Pst or mortality rate in case of infection with *B. cinerea*. Moreover, the quantification of bacterial population densities will further our knowledge on the potential nature of interactions between different bacteria *in planta* and hint towards the main drivers behind the protective effect.

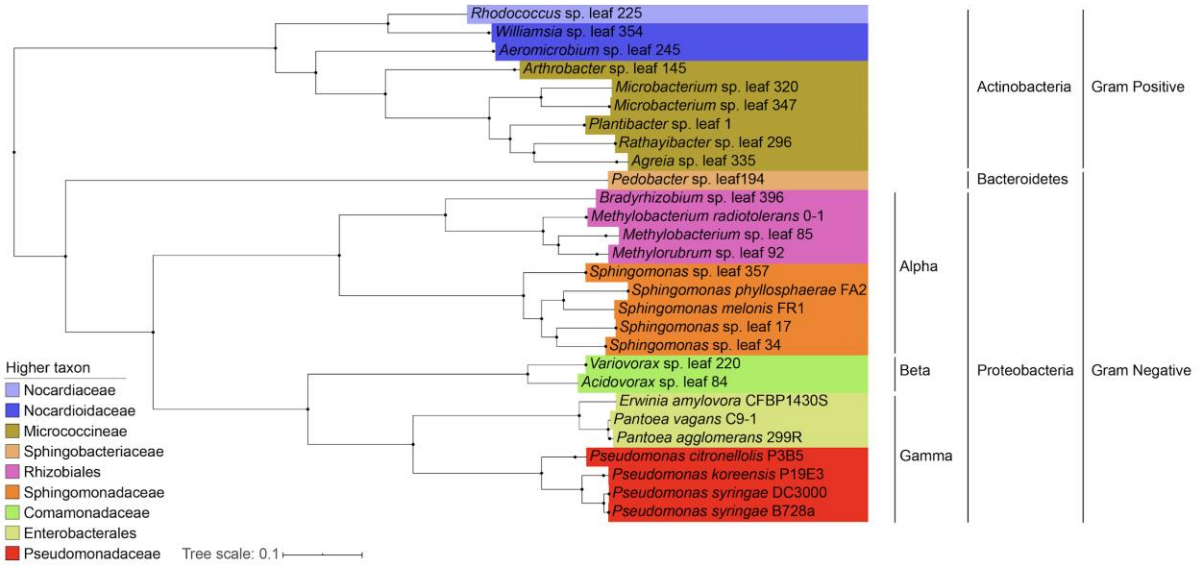


Figure 2. 1 Phylogenetic tree of bacteria employed in this project. The tree was produced by "Insert Genome Into SpeciesTree" app provided by Kbase website and modified in iTOL (Arkin et al., 2018; Letunic & Bork, 2007; Price et al., 2010). Scale bar represents the number of substitutions per amino acid.

2.2 Materials and Methods

2.2.1 Plant growth condition

Arabidopsis Col-0 seeds were grown on half strength Murashige-Skoog (½ MS, Duchefa, Netherlands) medium in a 24 well-plate system (Bio-one, Austria). ½ MS was adjusted to pH 5.9 and supplemented with 1% (w/v) plant agar (Duchefa, Netherlands). Each well contained 1 ml ½ MS medium. Seeds were surface sterilized with 70% ethanol supplemented with 0.1% Triton X-100 (Sigma, USA) for 1 min followed by 10% bleach supplemented with 0.1% Triton X-100 for 12 min. The seeds were washed three times with sterile ddH_2O and then stratified at 4 °C for 2 days. After stratification, one seed was sown in each well. Seedlings were grown under short day conditions (8-16 h dark-light shift) at 22 °C, 85% relative humidity, and 150-200 µmol photons m^{-2} s^{-1} in a CMP6010 growth chamber (Conviron, Canada). The 24-well plates were sealed with micropore tape (3M, USA) until non-pathogen inoculation. Afterwards, parafilm (Bemis, USA) was used instead of micropore tape in order to maintain a high humidity condition. After bacterial or fungal treatment, the 24-well plates were shuffled randomly every 2 days.

2.2.2 Non-pathogen and pathogen inoculation of plants

The biotrophic pathogen *Pseudomonas syringae* pv*. tomato* DC3000::Tn7-145/2 (Pst, tagged with the constitutively expressed the mScarlet gene coding for a red fluorescent

protein and a Gentamicin resistance gene) (Miebach et al., 2020; Schlechter et al., 2018), was grown on King's B (HIMEDIA LABORATORIES, India) agar plates and incubated at 30 °C. The necrotrophic pathogen, *B. cinerea* (strain ICMP16619, Landcare, New Zealand), was subcultured on Potato dextrose agar (HIMEDIA LABORATORIES, India) and incubated at room temperature 20 days before spores were harvested. Spores were collected and washed using 0.8% NaCl and filtered through cotton to remove hyphae. All bacterial strains used in this study are listed in Table 2.1. All non-pathogenic bacteria were cultured on R2A (HIMEDIA LABORATORIES, India) agar and incubated at 30 °C. Bacterial cells were collected and washed twice to remove media contaminations. To wash the cells, they were centrifuged at 4000 *g* for 5 min. Then the supernatant was discarded and the cells were resuspended in phosphate-buffered saline (PBS, 8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4 and 0.24 g/L KH_2PO_4, pH 7.4). The optical density (OD_{600nm}) was adjusted so that the suspension contained 6×10^6 colony forming units (cfu)/ml (Supplement table 2). For single inoculant experiments, 10 days old Arabidopsis seedlings were drop-inoculated with 5 µl of bacterial suspension at a density of 6×10^6 cfu/ml. 5 µl PBS were applied as mock treatment. For the biocontrol activity assays, 14 days old seedlings were inoculated with 5-10 µl of cell suspension of individual bacterial strains at a density of 6×10^6 cfu/ml. PBS was applied as a mock treatment. 5-10 µl Pst suspension (5×10^4 cfu/ml) were distributed onto each leaf of Arabidopsis 7 days after initial inoculation. For the necrotrophic pathogen, *B. cinerea*, spores were diluted in sterile Vogel buffer (15 g/L of sucrose, 3 g/L of Na-citrate, 5 g/L of K_2HPO_4, 0.2 g/L of $MgSO_4 \cdot 7H_2O$, 0.1 g/L of $CaCl_2 \cdot 2H_2O$, and 2 g/L of NH_4NO_3) to 10^5 cells/ml. Infection with *B. cinerea* spores was performed 10 days post non-pathogenic bacterial inoculation. To that end, 2 µl *B. cinerea* spore suspension was dropped onto one leaf of each plant.

Table 2. 1 Bacterial strains used in this study and their basic information with Arabidopsis.

Genus	Species	Strain	Arabidopsis Indigenous (Y/N)	Non-pathogen (Y/N)	Reference or source
Pantoea	*agglomerans*	299R*	N	Y	(Remus-Emsermann, Kowalchuk, et al., 2013)
Pantoea	*vagans*	C9-1	N	Y	(Brady et al., 2009)
Pantoea	*vagans*	PW	N	Y	**
Pseudomonas	*syringae*	B728a	N	N	(Feil et al., 2005)
Pseudomonas	*syringae*	DC3000::Tn7 -145/2*	N	N	(Miebach et al., 2020)
Pseudomonas	*citronellolis*	P3B5*	N	Y	(Remus-Emsermann et al., 2016)
Pseudomonas	*koreensis*	P19E3	N	Y	(Schmid et al., 2018)
Erwinia	*amylovora*	CFBP1430S	N	N	(Moreau et al., 2012)
Methylobacterium	*radiotolerans*	0-1*	N	Y	(Kwak et al., 2014)
Methylobacterium	sp.	leaf 85*	Y	Y	(Bai et al., 2015)
Methylorubrum	sp.	leaf 92*	Y	Y	(Bai et al., 2015)
Sphingomonas	*melonis*	Fr1*	Y	Y	(Innerebner et al., 2011)
Sphingomonas	*phyllosphaerae*	FA2*	N	Y	(Rivas et al., 2004)
Sphingomonas	sp.	leaf 17	Y	Y	(Bai et al., 2015)
Sphingomonas	sp.	leaf 34	Y	Y	(Bai et al., 2015)
Sphingomonas	sp.	leaf 357*	Y	Y	(Bai et al., 2015)
Bradyrhizobium	sp.	leaf 396	Y	Y	(Bai et al., 2015)
Acidovorax	sp.	leaf 84*	Y	Y	(Bai et al., 2015)
Variovorax	sp.	leaf 220	Y	Y	(Bai et al., 2015)
Agreia	sp.	leaf 335	Y	Y	(Bai et al., 2015)
Microbacterium	sp.	leaf 320	Y	Y	(Bai et al., 2015)
Microbacterium	sp.	leaf 347	Y	Y	(Bai et al., 2015)
Rathayibacter	sp.	leaf 296	Y	Y	(Bai et al., 2015)
Arthrobacter	sp.	leaf 145	Y	Y	(Bai et al., 2015)
Rhodococcus	sp.	leaf 225	Y	Y	(Bai et al., 2015)
Aeromicrobium	sp.	leaf245	Y	Y	(Bai et al., 2015)
Williamsia	sp.	leaf 354	Y	Y	(Bai et al., 2015)
Plantibacter	sp.	leaf 1	Y	Y	(Bai et al., 2015)
Pedobacter	sp.	leaf 194	Y	Y	(Bai et al., 2015)

* These bacteria have two phenotypes, wild type and fluorescent type. Wild type ones (except for Pst) were used in plant phenotypic screen experiments. Fluorescent ones (supplement table 1) were used in protoplast assays.
** *Pantoea vagans* PW is a white variant of C9-1 and misses a mega plasmid.

2.2.3 Bacteria antibiotic resistance test

In order to be able to determine the bacterial densities of the non-pathogenic bacteria and Pst during the biocontrol activity assay, I determined the antibiotic resistances of all applied bacterial strains beforehand, to check whether they could be separated using different antibiotics. Bacteria were subcultured as described above. Their optical density (OD_{600nm}) was adjusted to 1 before they were washed by centrifugation at 4000 *g* and resuspended in PBS. Finally, the optical density was adjusted to 0.5 and the bacterial suspensions were ten-fold serial diluted to a minimum of $OD_{600nm} = 0.5 \times 10^{-8}$. Afterwards, 3 µl of each dilution was spotted onto R2A agar supplemented with different antibiotics in square petri dishes (Table 2.2). These plates were incubated at 30 °C. After 3 days, cfu of bacteria were counted every

24 hours until no additional colonies emerged. The fold change of cfu on each plate compared with that on R2A plate was calculated based on following equation:

$$Fold\ Change\ = \frac{cfu\ number\ on\ antibiotic\ agar\ \times\ dilution\ factor}{cfu\ number\ on\ R2A\ agar\ \times\ dilution\ factor}$$

If the log-transformed value of fold change was between -1 to 1, the bacterium was evaluated as resistant to the respective antibiotic.

Table 2. 2 Antibiotics used in this study

Full name	Abbreviation	Working concentration (µg/ml)
Colistin	Col	10
Ampicillin	Amp	100
Gentamicin	Gm	10
Trimethoprim	Tri	100
Erythromycin	Em	100
Rifampicin	Rif	100
Chloramphenicol	Cm	20
Streptomycin	St	100
Kanamycin	Km	40
Tetracycline	Tet	15

2.2.4 Plant harvest and enumeration of phyllosphere bacteria

The recovery of bacterial cfu from the Arabidopsis phyllosphere was previously described by Miebach et al. (Miebach et al. 2020). Briefly, aboveground plant parts were carefully cut and put into PBS plus 0.02% (v/v) Silwet L-77 (Helena chemical company). Samples were processed in a bead ruptor tissue lyser by shaking twice for 5 minutes at 2.6 m/s (Omni Bead Ruptor 24). Afterwards, epiphytic and endophytic bacteria were collected. For experiments of bacteria colonisation capacity, four plants per 24-well plate were sampled after 16 days post inoculation (dpi, plant age = 30 days after sowing). The bacterial suspensions were serial diluted (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}). Then, about 3 µl droplets of each solution were stamped onto R2A agar. For dual-bacteria inoculation experiments, eight plants per 24-well plate were sampled 21 days post Pst infection (plant age = 35 days after sowing). Bacterial suspensions were serial diluted up to 10^{-5}. Then, about 3 µl droplets of each solution were stamped onto R2A agar supplemented with appropriate antibiotics in order to distinguish non-pathogenic bacteria and Pst (see 2.2.3). R2A agar supplemented with Rif or Gm was used to determine pathogen titre. Col was applied to select for most non-pathogens except for the genera *Pantoea* and *Pseudomonas*. For *Pantoea* strains, Em was used. For *Erwinia*

amylovora, Aeromicrobium sp. leaf 245 and *Bradyrhizobium* sp. leaf 396, only pathogen cfu could be determined since no antibiotic can be used to select for these strains.

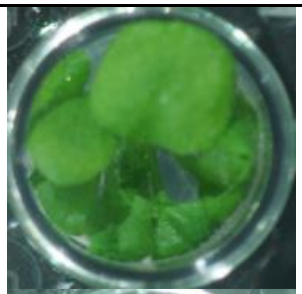
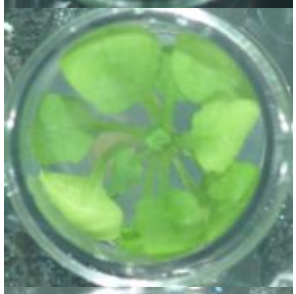
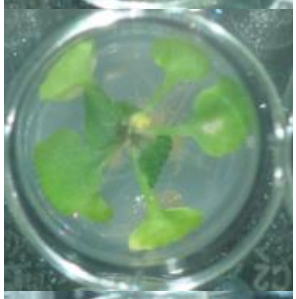
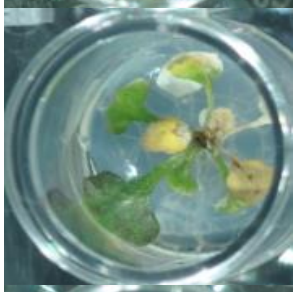
2.2.5 Plant leaf area measurement and statistical analysis for the experiments of bacteria colonisation on plants

Arabidopsis leaf area measurement was performed as described by Easlon and Bloom (Easlon & Bloom, 2014) using a photo stage and camera Nikon D90 (Nikon, Japan). Raw photos were taken at 14 days post inoculation. Fiji/ImageJ (version: 2.0.0-rc-54/1.52p) was used to process raw images and measure the leaf area (Schindelin et al., 2012). Graphpad Prism 8 and R (R Core Team, 2019) were used for plotting graphs, data manipulation and statistical analysis. An analysis of variance (ANOVA) was performed on the leaf area of each treatment for plant growth affected by individual strains. Regression analysis was performed by using “geom_smooth(method=’lm’)” function in R.

2.2.6 Disease index calculation for the experiments of testing the protective ability of non-pathogens

As a proxy of Pst pathogenicity, the disease index of Arabidopsis was determined after 20 days post infection. Raw images were acquired and the disease level of each plant was assessed. Disease levels were assessed using the arbitrary scale shown in Table 2.3. In terms of *B. cinerea* infection, raw images were acquired every 24 hours after *B. cinerea* infection. Then dead plants (90% leaf area turned brown) were visually counted to calculate the plant mortality rate of each bacterial treatment.

Table 2. 3 Arbitrary disease index scale for quantifying disease severity caused by Pst

Disease Index 1 (completely healthy)	
Disease Index 2 (less healthy but no disease symptom)	
Disease Index 3 (mild disease symptoms)	
Disease Index 4 (severe disease symptoms)	
Disease Index 5 (full chlorosis)	

2.3 Results

2.3.1 Effect of bacterial inoculation on plant growth and bacterial colonisation of Arabidopsis

Before dual-inoculation with Pst or *B.cinerea* on Arabidopsis, the ability of all non-pathogenic strains to colonise in the phyllosphere by leaf-inoculation method and the impact of non-pathogen colonisation on plant growth were tested. As shown in Table 2.4 and Fig 2.2, none of the bacterial strains except for Pst and *P. syringae* B728a had adverse effects on Arabidopsis growth. Additionally, none of the tested strains showed a significant plant growth-promotion effect.

Most bacterial strains except for *Sphingomonas* sp. leaf 34, *Methylobacterium* sp. leaf 85 and *Bradyrhizobium* sp. leaf 396 successfully colonised leaves after 10 days post inoculation (Fig 2.2). In general, successful colonizers grew to a relatively high abundance in the phyllosphere, reaching up to 10^{10} cfu per gram plant material (fresh weight) (Fig 2.2). Overall, the presence of non-pathogenic bacteria did not affect the growth and development of the plant host.

Table 2. 4 Comparisons of mean leaf area of Arabidopsis inoculated with non-pathogenic bacteria to mock inoculated plants.

Comparisons	Difference of mean leaf area (cm^2)	P value*	Significance#
Mock vs. *Pseudomonas syringae* DC3000	0.16	0.30	ns
Mock vs. *Pseudomonas syringae* B728a	0.22	0.01	*
Mock vs. *Sphingomonas melonis* FR1	0.06	1.00	ns
Mock vs. *Sphingomonas phyllosphaerae FA2*	0.07	1.00	ns
Mock vs. *Sphingomonas* sp. leaf 34	0.03	1.00	ns
Mock vs. *Sphingomonas* sp. leaf 17	0.09	0.99	ns
Mock vs. *Sphingomonas* sp. leaf 357	0.05	1.00	ns
Mock vs. *Methylobacterium radiotolerans* 0-1	0.10	0.98	ns
Mock vs. *Methylobacterium* sp. leaf 92	0.09	0.99	ns
Mock vs. *Methylobacterium* sp. leaf 85	0.08	1.00	ns
Mock vs. *Pantoea agglomerans* 299R	0.03	1.00	ns
Mock vs. *Pantoea vagans* C9-1	-0.03	1.00	ns
Mock vs. *Pantoea vagans* PW	0.14	0.45	ns
Mock vs. *Pseudomonas citronellolis* P3B5	-0.03	1.00	ns
Mock vs. *Pseudomonas koreensis* P19E3	-0.11	0.97	ns
Mock vs. *Erwinia amylovora* CFBP1430S	-0.10	0.98	ns
Mock vs. *Bradyrhizobium* sp. leaf 396	-0.14	0.51	ns
Mock vs. *Acidovorax* sp. leaf 84	0.11	0.93	ns
Mock vs. *Variovorax* sp. leaf 220	0.05	1.00	ns
Mock vs. *Agreia* sp. leaf 335	0.04	1.00	ns
Mock vs. *Microbacterium* sp. leaf 320	0.03	1.00	ns
Mock vs. *Microbacterium* sp. leaf 347	0.01	1.00	ns
Mock vs. *Rathayibacter* sp. leaf 296	-0.01	1.00	ns
Mock vs. *Arthrobacter* sp. leaf 145	0.02	1.00	ns
Mock vs. *Rhodococcus* sp. leaf 225	0.06	1.00	ns
Mock vs. *Aeromicrobium* sp. leaf 245	0.05	1.00	ns
Mock vs. *Williamsia* sp. leaf 354	0.08	1.00	ns
Mock vs. *Plantibacter* sp. leaf 1	0.06	1.00	ns
Mock vs. *Pedobacter* sp. leaf 194	0.06	1.00	ns

*ANOVA comparison of all treatments against mock inoculated controls; #Results were evaluated by Tukey's Honest Significant Difference test. * p = 0.001 to 0.01, 'ns' = no significant difference.

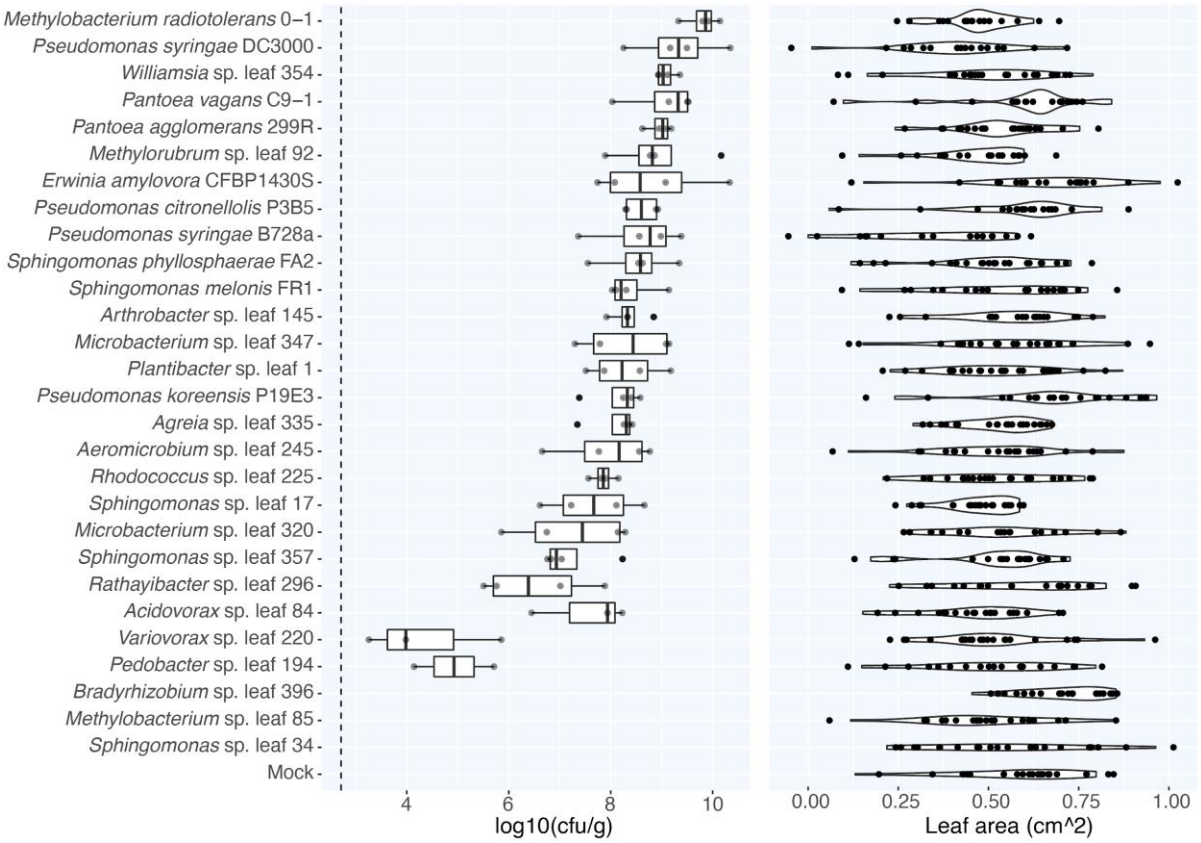


Figure 2. 2 Bacterial density in the phyllosphere (left) and plant leaf area (right). (left) Bacterial density recovered from leaves after 10 dpi (log-transformed cfu per gram of leaf fresh weight). Centerlines in box plots represent the 50th percentile and each box shows the interquartile range (four random plant samples). Outlier dots represent values over 1.5 times the interquartile range beyond either end of the box. The bacterial population data only includes values above the limit of detection (log10(cfu/g) > 2.71) indicated by the dotted line. Y axis labels were sorted by the average bacterial population density (right). The violin plot of the leaf area after leaf inoculation with individual bacterial strains in Table 2.1 after 9 dpi. Dots represent the leaf area of individual samples.

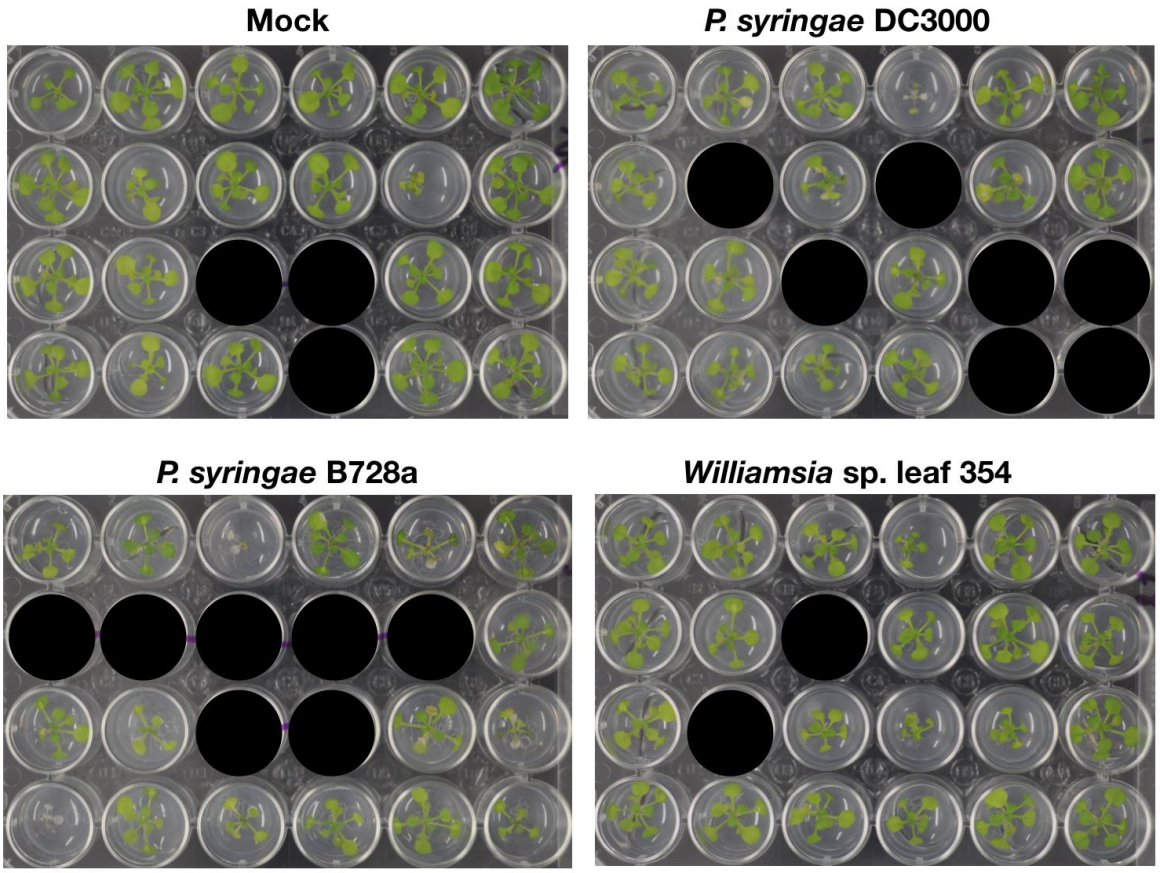


Figure 2. 3 Example photographs of axenic, P. syringae-infected and Williamsia sp. leaf 354-inoculated Arabidopsis after 10 dpi. PBS was applied as a mock treatment. 6×10^6 cfu/ml bacterial solution was leaf inoculated on 14 days old Arabidopsis plants. Plants covered by black circles were excluded from the experiment as these plants did not germinate or were developmentally retarded.

2.3.2 Bacteria antibiotic resistance screen

As this research frequently dual-inoculated non-pathogenic bacteria and Pst on leaves, antibiotics were used to select non-pathogens and pathogens for colony quantification. Therefore, the antibiotic resistances of bacterial strains were determined. The pathogen Pst has strong resistance against Gm, Tri, Rif and Cm (Table 2.5). On the other hand, most non-pathogenic bacteria were strongly resistant to Col except for genus *Pantoea, Pseudomonas* and *Erwinia* and one species in genus *Aeromicrobium* (Table 2.5). As a result, Col was employed to select for most non-pathogenic strains, while Pst was selected on agar supplemented with Rif or Gm. Em was used to select for *Pantoea* strains. Amp was used to select for the three other *Pseudomonas* strains.

Table 2. 5 Antibiotic resistances of the bacterial strains listed in Table 2.1.*

Strain	Col	Amp	Gm	Tri	Em	Rif	Cm	St	Km	Tet
Pantoea agglomerans 299R					-2	1				
Pantoea vagans C9-1					-1					
Pantoea vagans PW					0					
*Pseudomonas syringa*e B728a		-2		0	-2	-1	0			
Pseudomonas citronellolis P3B5	-2	-1		0	-5		0			
Pseudomonas koreensis P19E3	-6	-2		0	-5		0			
Erwinia amylovora CFBP1430S				-4		-5	-3			
Methylobacterium radiotolerans 0-1	0		0	0	0		0			
Methylobacterium sp. leaf 85	0	0		0			0			
Methylobacterium sp. leaf 92	0		0	0	0		0			
Sphingomonas melonis FR1	0	0			0			0		
Sphingomonas phyllosphaerae FA2	0	-5		-3	0			0		
Sphingomonas sp. leaf 17	0		0	-5	-5					
Sphingomonas sp. leaf 34	0		-3	-2						
Sphingomonas sp. leaf 357	0	-1		-2				-1		
Bradyrhizobium sp. leaf 396	-5	-5	0	0			-1		0	
Acidovorax sp. leaf 84	0	0	0	0						
Agreia sp. leaf 335	0									
Microbacterium sp. leaf 320	0		0							
Microbacterium sp. leaf 347	0		-3	0						
Rathayibacter sp. leaf 296	0			0						
Arthrobacter sp. leaf 145	0		0	-6					-6	
Rhodococcus sp. leaf 225	0			0						
Aeromicrobium sp. leaf 245	-6					0				
Williamsia sp. leaf 354	0			0						-1
Plantibacter sp. leaf 1	-1									
Pedobacter sp. leaf 194	0	-3	0						0	
Pseudomonas syringae DC3000			0	0		0	0			

*The numbers in the table are log10 transformed of bacterial colony number and their respect difference value compared with mock treatment (R2A agar). The difference value between -1 to 1 is shaded in light green colour, which means that strain has strong resistance to the antibiotic. Cells that have a difference value lower than -1 or blank (no colony growing on antibiotic plates) means bacterial strains are highly susceptible to the antibiotic.

2.3.3 Plant-protective effect of non-pathogens against *Pseudomonas syringae* DC3000

In order to test the plant-protective effect of non-pathogens against the biotrophic pathogen Pst, plants were pre-colonised by the individual competitor strains before they were infected with Pst. Three weeks after Pst infection, the disease index of the plants were visually rated. In addition, non-pathogen and Pst cell numbers (eight random individual plants for every treatment) in the phyllosphere parts were also determined. Two independent experiments were performed. However, due to an experimental error, bacteria cells from some plants were not successfully harvested. For instance, Pst populations of mock pre-treatment plants in the first experiment were under the limit of detection. Potentially, Pst cells were killed during the sonication process since overheating of the water bath. Also, the prolonged and continuous use of the bead ruptor might influence the stability of the machine. Based on the results of the two experiments, *Aeromicrobium* sp. leaf 245, *Bradyrhizobium* sp. leaf 396, *Erwinia amylovora*, all Methylotrophs, *Pantoea agglomerans* 299R, *Rhodococcus* sp. leaf 225, *Variovorax* sp. leaf 220, and *Williamsia* sp. leaf 354, were rated as nonprotective strains (Table 2.6). *Agreia* sp. leaf 335, *Arthrobacter* sp. leaf 145, *Mycobacterial* strains, *Pantoea vagans* C9-1, *Pseudomonas koreensis* P19E3, *Pseudomonas syringae* B728a and *Rathayibacter* sp. leaf 296, were rated as intermediate protective strains as the ranks of these groups were in the middle based on their average disease index. Also, the plant phenotypes were less predictable with these strains such as *Microbacterium* sp. leaf 320 (Table 2.6). *Acidovorax* sp. leaf 84, *Pedobacter* sp. leaf 194, *Plantibacter* sp. leaf 1, *Pseudomonas citronellolis* P3B5 and *Sphingomonas* species were rated as fully protective bacteria. Most protective strains were Gram negative strains (8 out of 9 protective strains) with only *Plantibacter* sp. leaf 1 belonging to Gram positive bacteria.

Table 2. 6 Average disease index of different plant groups

Strain	First experiment	Second experiment	Mean of two experiments	Protective ability
Sphingomonas phyllosphaerae FA2	1.57	2.43	2.00	+
Pedobacter sp. leaf 194	2.00	2.39	2.19	+
Sphingomonas melonis FR1	1.78	2.65	2.21	+
Acidovorax sp. leaf 84	2.13	2.33	2.23	+
Sphingomonas sp. leaf 17	1.89	2.76	2.33	+
Pseudomonas citronellolis P3B5	2.26	2.56	2.41	+
Sphingomonas sp. leaf 34	2.11	2.78	2.44	+
Sphingomonas sp. leaf 357	2.12	2.78	2.45	+
Plantibacter sp. leaf 1	2.17	2.75	2.46	+
Agreia sp. leaf 335	2.40	2.53	2.46	±
Microbacterium sp. leaf 320	1.94	3.00	2.47	±
Microbacterium sp. leaf 347	2.45	2.53	2.49	±
Arthrobacter sp. leaf 145	2.23	2.84	2.53	±
Rathayibacter sp. leaf 296	2.55	2.52	2.54	±
Pantoea vagans C9-1	2.33	2.78	2.56	±
Pseudomonas koreensis P19E3	2.37	2.85	2.61	±
Pseudomonas syringae B728a	2.58	2.73	2.66	±
Erwinia amylovora CFBP1430S	2.81	2.82	2.82	-
Variovorax sp. leaf 220	2.76	3.00	2.88	-
Methylobacterium radiotolerans 0-1	2.69	3.11	2.90	-
Pantoea agglomerans 299R	2.87	3.13	3.00	-
Bradyrhizobium sp. leaf 396	3.33	2.95	3.14	-
Methylorubrum sp. leaf 92	2.82	3.63	3.22	-
Rhodococcus sp. leaf 225	3.13	3.57	3.35	-
Axenic plants	3.36	3.50	3.43	-
Aeromicrobium sp. leaf 245	3.35	3.75	3.55	-
Williamsia sp. leaf 354	3.30	3.94	3.62	-
Methylobacterium sp. leaf 85	3.50	3.83	3.67	-

Without considering the values below the limit of detection, most plants with disease index 4 or 5 hosted high abundance of Pst (10^9 cfu/g) (Fig 2.4A). However, Pst populations on plants with disease index 1, 2 and 3 did not show a distinct difference, ranging between 10^4 to 10^9 cfu/g (Fig 2.4A). Plants inoculated with *Microbacterium* sp. leaf 320 exhibited less predictable phenotypes in the two experiments. The average disease index of these plants in the first experiment is 1.94 but 3.00 in the second experiment (Table 2.6). Overall, plant groups follow similar trends in both experiments, which indicates a good reproducibility.

In addition to plant phenotypes, the proliferation of non-pathogens and Pst in the phyllosphere gives insight on how protective strains confer their protection on microbial community levels. The results show that the bacterial population largely depends on their taxa level. Plants pre-colonised with Gram positive strains, most were Norcadioidaceae strains, seem to host high Pst population densities, irrespective of the non-pathogen population density (Fig 2.5A). However, the population density of Gram negative strains shows a correlation with the Pst population density (R^2=0.16, p=3.9^{-6}, Fig 2.5A).

After simplifying the data to a lower taxonomic level, the clusters of the relationships of non-pathogen population and Pst population densities are easier to interpret. Bacterial populations of the same subphylum level showed similar patterns (Fig 2.5B, 2.6): Pst populations from plants with Comamonadaceae strains were all lower than 10^6 cfu/g. Plants pre-inoculated with Rhizobiales host both high numbers of non-pathogens and Pst except for *Bradyrhizobium* sp. leaf 396 (Fig 2.6A). Even though *Bradyrhizobium* sp. leaf 396 was not evaluated as a protective strain, this strain could reduce pathogen proliferation whilst not diminish disease symptoms. On the other hand, Sphingomonadaceae-colonising plants have a wider range of non-pathogen and Pst population densities (10^4 to 10^{10} cfu/g, Fig 2.6A). Plants with Pseudomonadaceae host a wide range of Pst population densities ranging from 10^4 to 10^9 cfu/g (Fig 2.6B). The population densities of Pseudomonadaceae strains were ranging from 10^7 to 10^{11} cfu/g (Fig 2.6B). Pst cell numbers were in the range of 10^6 to 10^5 cfu/g from plants with Enterobacterales while the population sizes of Enterobacterales strains ranged between 10^5 to 10^{10} cfu/g (Fig 2.6B).

Regression analysis on species level was performed to evaluate correlations between non-pathogen population size and Pst cell numbers. For most *Sphingomonas* strains, their population sizes were not highly correlated with Pst population densities (R^2 <0.6 and p < 0.29, Fig 2.7A). Especially for *Sphingomonas phyllosphaerae* FA2, R^2 value was 0.08 (Fig 2.7A). The other three protective strains and *Sphingomonas* sp. leaf 357, exhibited a higher correlation between non-pathogen population densities and Pst population densities (R^2 >0.63 and p < 0.042, Fig 2.7A). For Methylotrophs, Pst population sizes were moderately correlated with Methylotroph populations (R^2 > 0.59 and p < 0.025, Fig 2.7B).

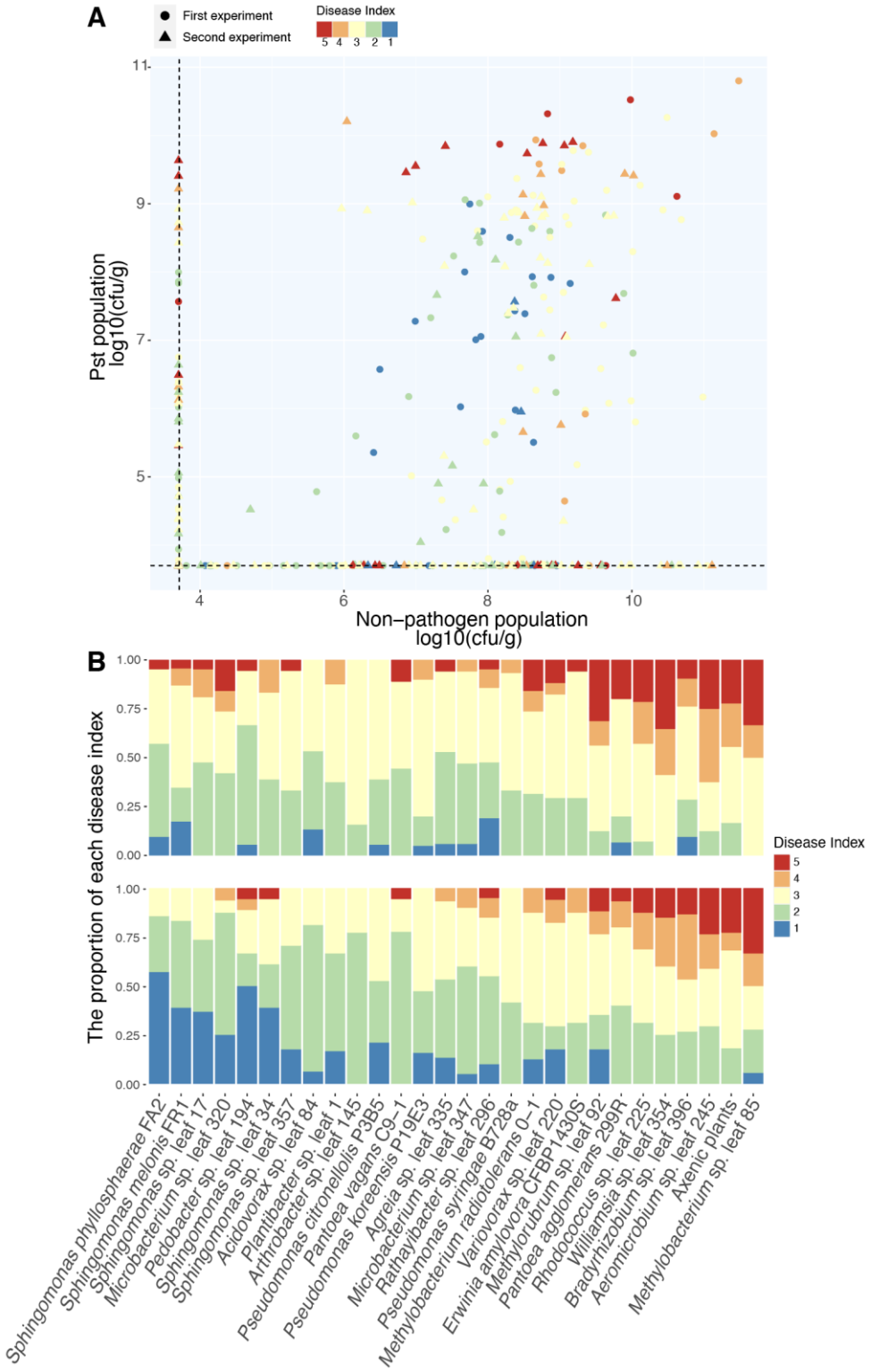


Figure 2. 4 Bacteria population densities from plant phyllosphere and plant phenotypic summary of each group. (A) Correlation between non-pathogen population and Pst population density. The plot shows the non-pathogen population and Pst population density of individual plants at 21 days post Pst infection. Cfu were log-transformed and normalised per gram of leaf material. Points are coloured by their corresponding disease index. Data included results from two experiments, indicated by different shapes. The bacterial population data only includes values above the limit of detection (log10(cfu/g) > 3.71). (B) Distribution of plant disease index in each treatment. Individual plants of each group were visually rated from disease index 1 to 5. Bacterial strain names are sorted based on their average disease index of the first experiment. (B Top) Second experiment result, (B Bottom) first experiment result.

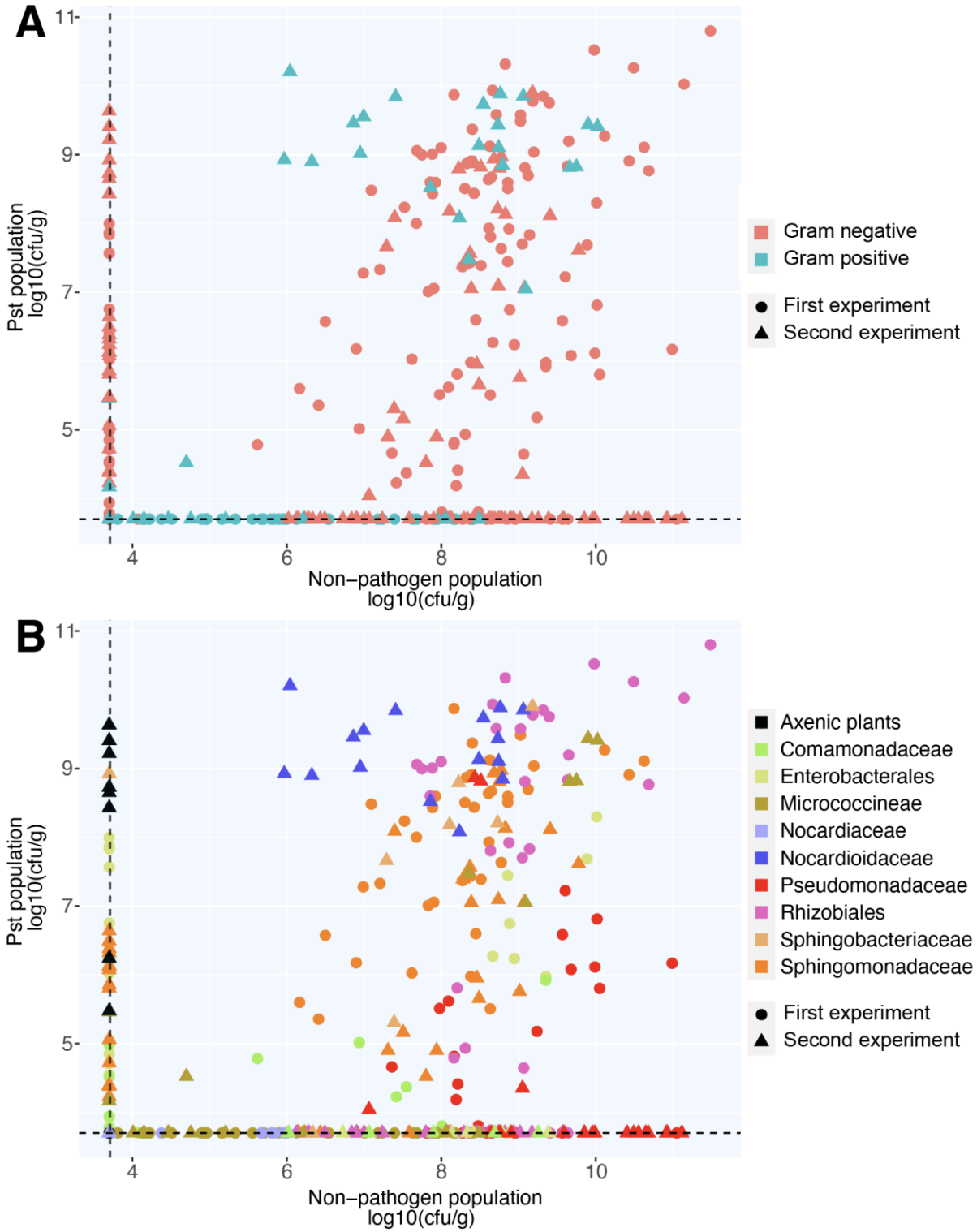


Figure 2. 5 The correlation of the non-pathogen population and Pst population density at different taxonomic ranks. Non-pathogen population and Pst population density of individual plants at 21 days post Pst infection. Cfu were log-transformed and normalised per gram of leaf material. Data includes results from two experiments, indicated by different shapes. The bacterial population data only includes values above the limit of detection (log10(cfu/g) > 3.71). (A) Points are coloured by Gram positive or negative. (B) Points are coloured by different taxon levels.

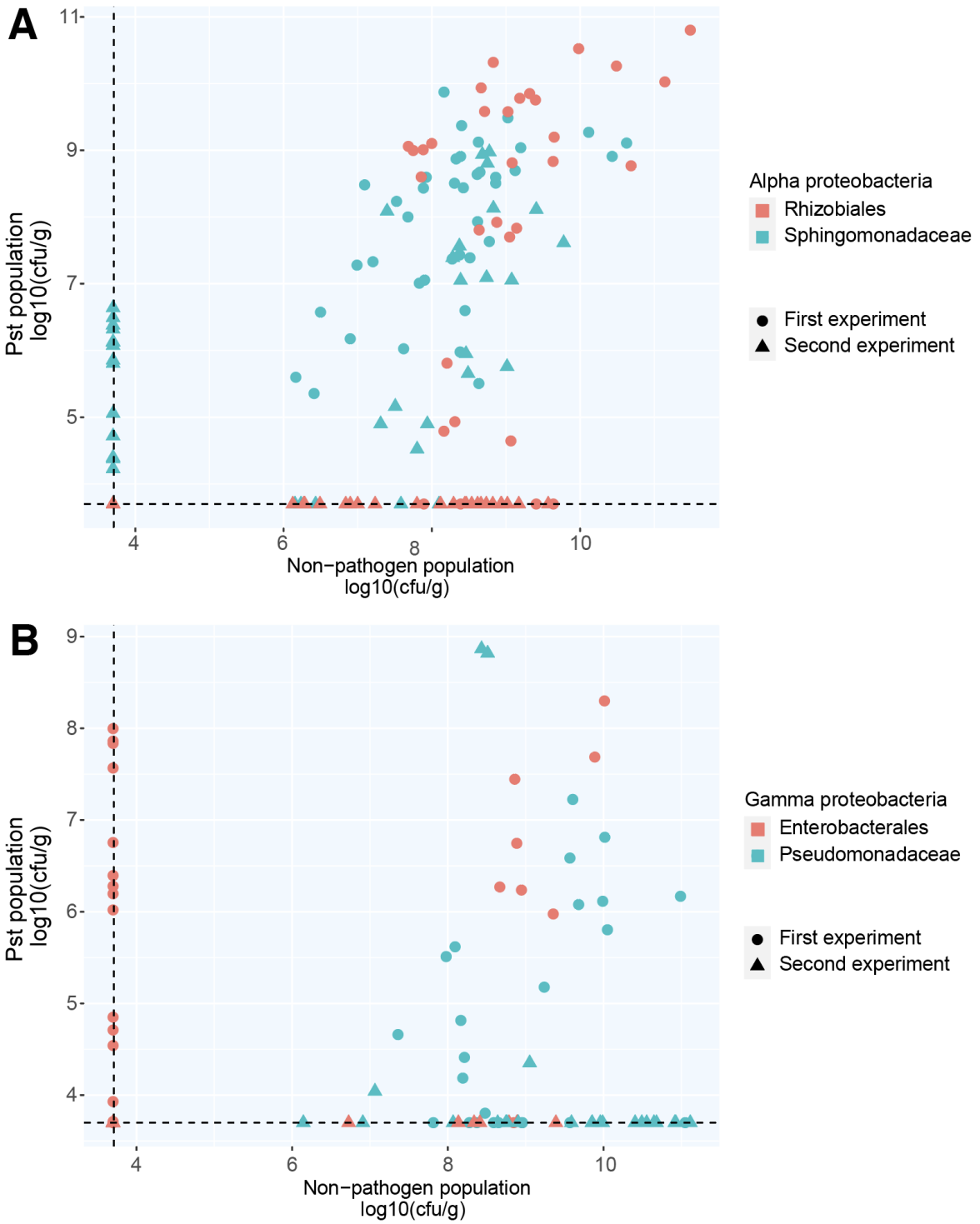


Figure 2. 6 The correlation of the non-pathogen population and Pst population density from alpha proteobacteria and gamma proteobacteria. The plot shows the non-pathogen population and Pst population density of individual plants at 21 days post Pst infection. cfu were log-transformed and normalised per gram of leaf material. Data included results from two experiments, indicated by different shapes. The bacterial population data only includes values above the limit of detection (log10(cfu/g) > 3.71). (A) The comparison between Rhizobiales and Sphingomonadaceae. (B) The comparison between Enterobacterales and Pseudomonadaceae.

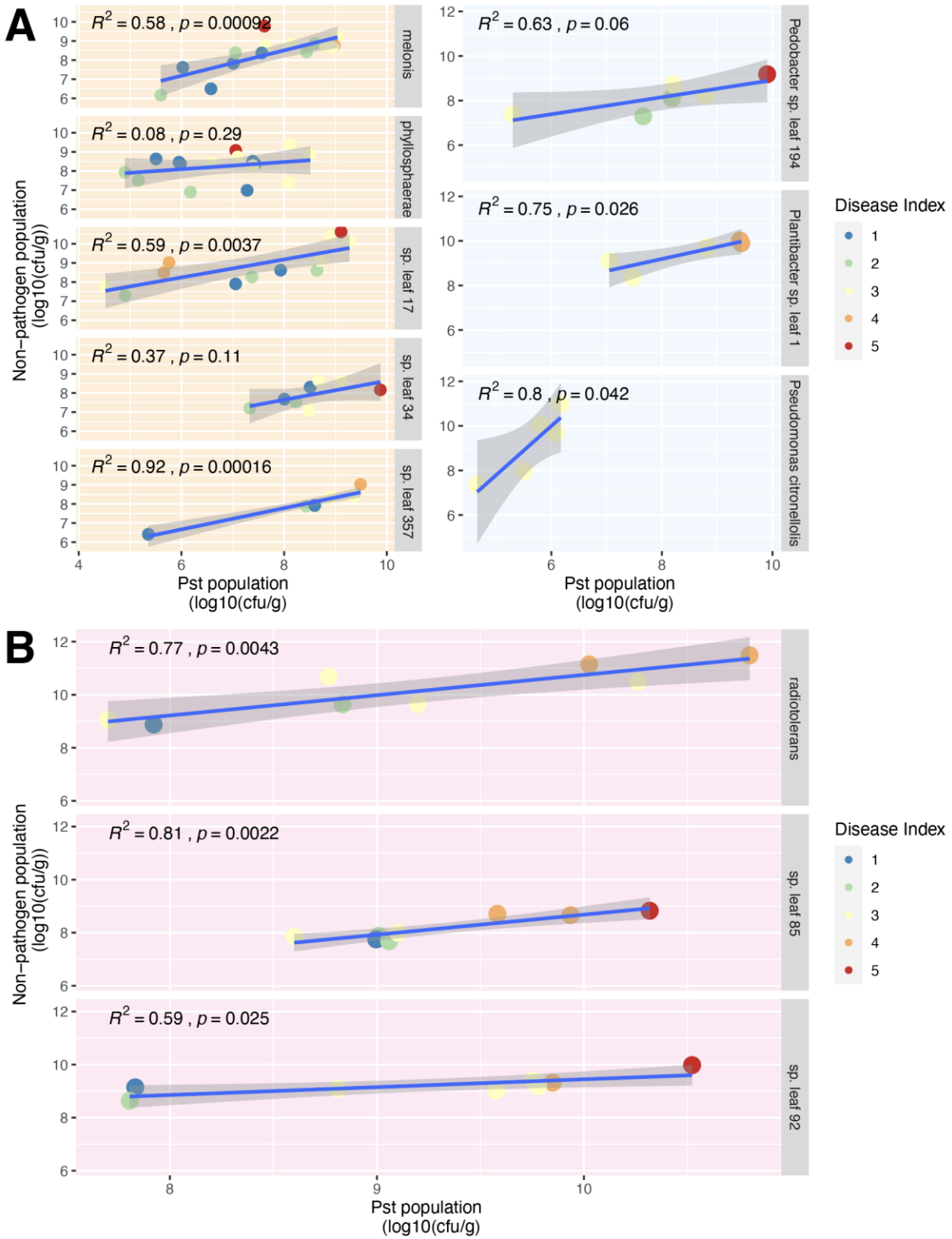


Figure 2. 7 Regression analysis of the non-pathogen population and Pst population on the strain levels. (A) *Sphingomonas* strains (yellow background), other three protective strains (blue background), (B) Methylotrophic strains (pink background). The plot shows the non-pathogen population and Pst population density of individual plants after 21 days post Pst infection. Cfu were log-transformed and normalised per gram of leaf material. Data included results from two experiments. Points are coloured by their corresponding disease index and represent the bacterial density of individual samples.

2.3.4 Plant-protective ability of non-pathogens against *Botrytis cinerea*

Unlike Pst infection, *B. cinerea* infection on Arabidopsis is more intense and deadly. As a result, the plant mortality rate was applied as an indicator of bacterial protective ability. 14-day-old Arabidopsis plants were leaf inoculated with individual bacterial strains. After 10 days, *B. cinerea* spore suspensions were dropped onto one single leaf of each plant. From 48 hours of *B. cinerea* infection, plant phenotypes were visually determined every 24 hours to count the proportion of dead plants. After 72 hours mortality could be detected even though some leaves already showed severe lesions before (Fig 2.9).

Bacterial strains which were capable of keeping a mortality rate below 0.125 at 96 hours post infection and below 0.375 at 120 hours post infection were rated as protective strains. Thus, *Arthrobacter* sp. leaf 145, *Pseudomonas syringae* DC3000, *Pseudomonas syringa*e B728a, *Pantoea vagans* PW, *Pantoea vagans* C9-1, *Pantoea agglomerans* 299R, *Rhodococcus* sp. leaf 225, *Sphingomonas* sp. leaf 17, *Sphingomonas phyllosphaerae* FA2 and *Sphingomonas melonis* FR1 were defined as protective strains against *B. cinerea*. However, most plants died between 96 to 120 hours (Fig 2.8). Notably, plants colonised by *Sphingomonas* sp. leaf 34 were more sensitive to *B. cinerea* infection, as 90% plants dead at 120 hours were already dead at 96 hours (Fig 2.8). On the other hand, *Pantoea agglomerans* 299R, *Pantoea vagans* C9-1 and *Pseudomonas syringae* B728a not only kept all plants alive but also significantly reduced the lesion damage area caused by *B. cinerea* (Fig 2.9).

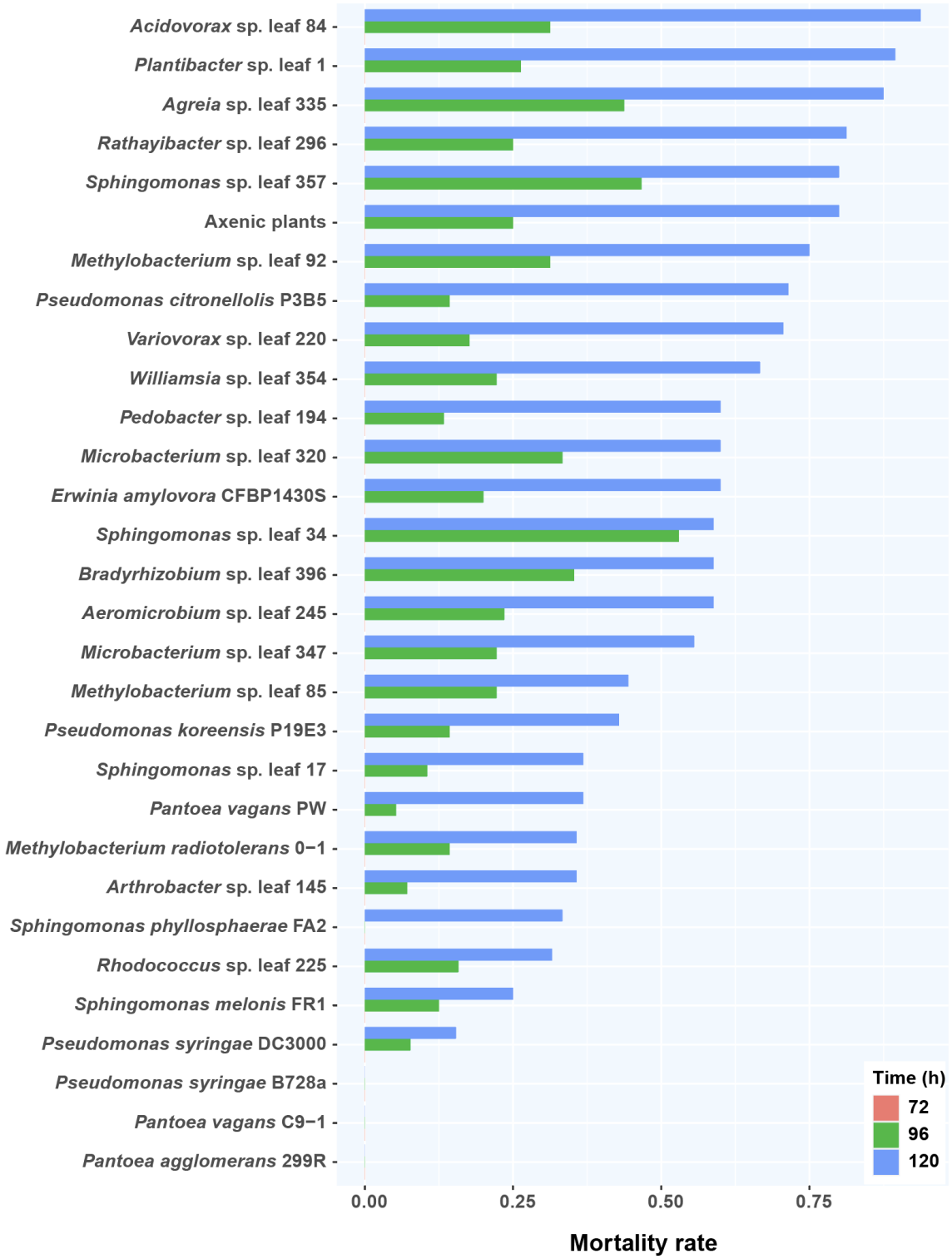


Figure 2. 8 Plant mortality development infected by B. cinerea. The plot shows mortality of plants that were pre-colonised by individual bacterial strains. Sample size varied from 12 to 20. If 90% of the leaf area of a plant turned brown, as it was defined as dead. All plants were still alive after 72 hours post *B. cinerea* infection.

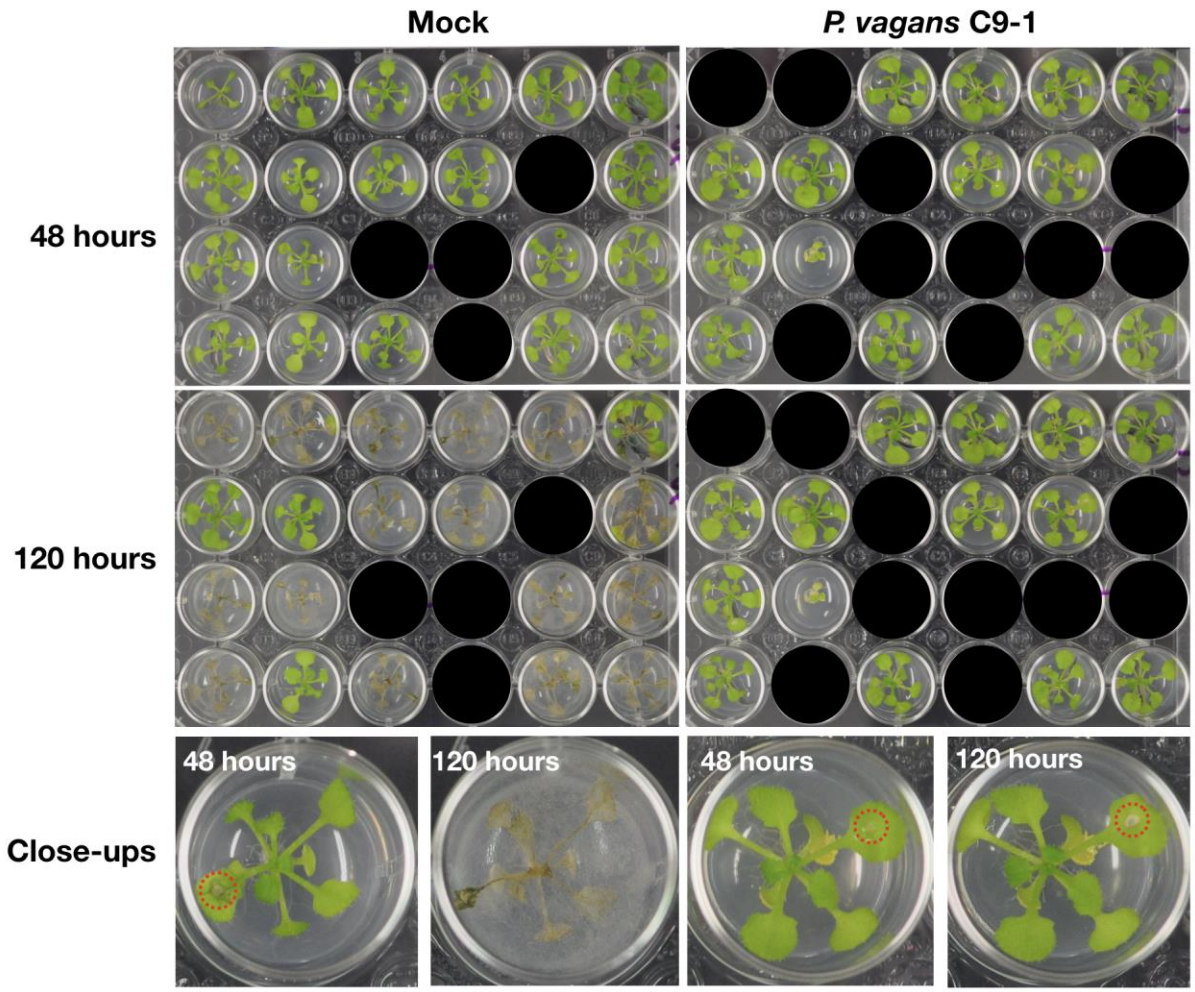


Figure 2. 9 Example photographs of plant phenotype development after 48 hours and 120 hours post *B. cinerea* infection. The plants were leaf inoculated with *P. vagans* C9-1 or treated by PBS prior to *B. cinerea* spores application. Plants covered by black circles were excluded from the experiment pre inoculation as these plants did not germinate or were developmentally retarded. The lesions are highlighted by red circles.

2.4 Discussion

2.4.1 Bacterial colonisation on Arabidopsis

The first aim of this study was to screen the bacteria colonising capacity after leaf inoculation. In preliminary experiments, different inoculation methods were tested including seed inoculation by mixing Arabidopsis seeds with bacterial suspension solution, pipetting droplets of bacterial suspension solution onto seeds and distributing bacterial suspension onto leaves by pipetting. After seed inoculation, *P. syringae* B728a prevented Arabidopsis seeds from germinating by forming colonies around the seeds. This may be the result of nutrition-acquisition from seeds by bacteria. Furthermore, *Sphingomonas* sp. leaf 34 failed to colonise leaves after seed inoculation, which might infer that certain bacteria could merely survive in the phyllosphere. In comparison to seed inoculation, leaf inoculation resulted in

sizable populations of most bacterial strains on leaves. However, *Sphingomonas* sp. leaf 34, *Methylobacterium* sp. leaf 85 and *Bradyrhizobium* sp. leaf 396 did not show cfu above the limit of detection, this may be explained by the slow growth rate of these bacteria which is also apparent on agar media. Therefore these strains might need more time to grow to a sizable population density on plants. After inoculating Pst, the population sizes of non-pathogens did not change significantly which indicates that leaf inoculation method and the time (seven days) for preemptive colonisation allowed non-pathogens to reach their carrying capacity in the phyllosphere (Supplement Table 3).

Another aim of this study was to determine the effect of bacterial monocultures on Arabidopsis growth. It was reported that non-pathogenic bacteria can promote plant growth (e.g. producing plant growth-stimulating factors, modulating plant hormone production) (Abanda-Nkpwatt et al., 2006; Adhikari et al., 2001; Innerebner et al., 2011). Thus, it is important to understand whether plant growth is significantly impacted by non-pathogens before pathogen infection takes place. All plants inoculated with non-pathogenic bacteria exhibited comparable, not significantly different leaf areas. Interestingly, even though Vinatzer et al. (2006) claimed that the bean pathogen *P. syringae* B728a does not cause disease and even has growth-promoting effects on Arabidopsis, plants did show disease symptoms and had lower mean leaf area compared with mock treatment in this study (Fig 2.3). This could result from the increasing pathogenesis due to the fact that air humidity in the 24-well plate system is maintained at a relatively high level (Xin et al., 2016). Overall, these results add to the confidence that the subsequent results are not influenced by plants that showed significantly larger or smaller growth as a result of positive or negative impact by pre-inoculated bacteria.

2.4.2 The experimental settings of *in planta* assay

According to Innerebner et al., the carrying capacity of 35-day-old Arabidopsis for *S. melonis* and Pst was around 1.5×10^9 cfu/g and 10^{10} cfu/g respectively (Innerebner et al., 2011). Their study used relatively high concentrations of non-pathogens and inoculated lower concentration of Pst (5×10^4 cfu/ml). Since the reproductive success of secondary colonisers largely depends on the population size of pre-colonisers in the phyllosphere (Remus-Emsermann, Kowalchuk, et al., 2013), in my project, non-pathogen and Pst concentration for inoculation were adjusted to make two bacteria both have sufficient chances to proliferate on plant leaves. Non-pathogen and Pst concentrations were adjusted to 6×10^6 cfu/ml (equivalent to about 3×10^4 cfu/g on 14 days old seedlings) and 5×10^4 cfu/ml (equivalent to

about 2×10^4 cfu/g on 21 days old seedlings). For *B. cinerea* infection, about 200 spores were dropped onto each plant which was enough to cause severe fungal infection. In order to help bacteria establish a relatively high abundance on plant leaves, parafilm was used to maintain a high humidity in the 24-well plate system. By using this protocol, it was possible to produce reproducible results and suit all non-pathogenic strains in a consistent environment.

For plants infected with Pst, the plant disease severity was visually scored based on disease symptoms of each plant, as described by Vogel et al (Vogel et al., 2012). The sample size varied from 16 to 20, as some plants did not grow or were developmentally retarded. Those were excluded at the start of the experiment before non-pathogen inoculation to minimise the variation of plant growth. Plants might be rated as the same disease index, however their spectral characteristics may be different due to modifications in leaf pigments, permeability or the appearance of pathogen-specific structures resulted from different types of plant-microbe interactions (Fig 2.10) (Gamon & Surfus, 1999; Mahlein et al., 2012). Especially for plants with disease index 1, 2 and 3, the plant phenotype and morphology were ambiguous and harder to distinguish (Table 2.3, Fig 2.10). In the future, hyperspectral imaging could be employed as an additional tool to quantify the early stages of disease severity. This technology is well suitable for the assessment for pre-symptomatic stages of plant diseases as it uses pixel-wise attribution of spectral signatures (Lowe et al., 2017; Mahlein et al., 2012). Hyperspectral imaging can be used not only in the quantification of disease severity caused by Pst, but also to detect the early effect of *B. cinerea* infection such as tiny spots or small-scale lesion by the hyperspectral information in spatial resolution (Mahlein et al., 2012).

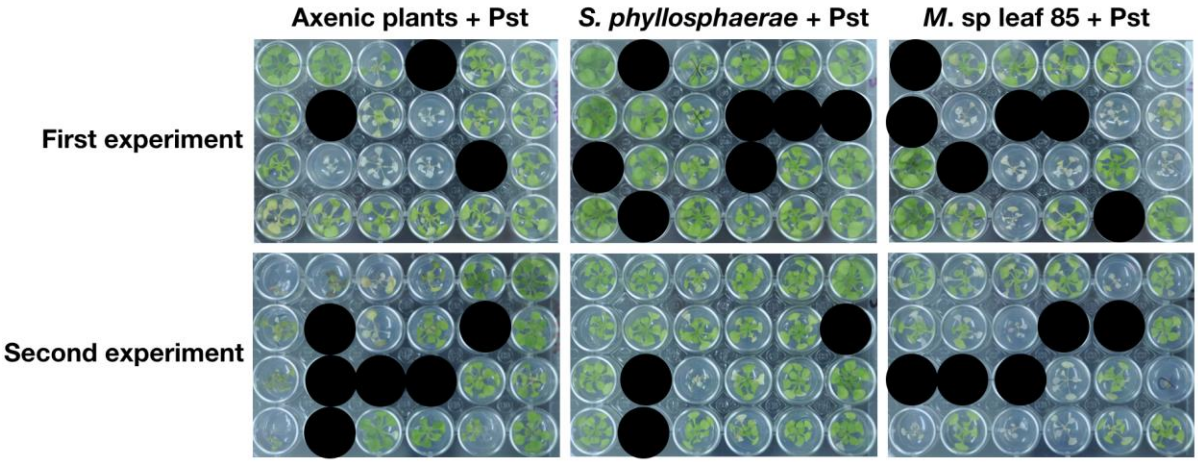


Figure 2. 10 Example photographs of plant phenotype from representative groups at 20 days post Pst infection. PBS, S. phyllosphaerae FA2 or M. sp. leaf 85 was applied by leaf inoculation on 14 days old plants. 21 days post inoculation, plants were challenged by Pst and imaged 20 days post infection. Plants covered by black circles were excluded from the experiment pre inoculation as these plants did not germinate or were developmentally retarded.

2.4.3 Plant-protection against *Pseudomonas syringae* DC3000 by non-pathogenic preemptive colonisation

By screening a diverse set of non-pathogenic bacterial taxa, the protective bacterial groups against Pst were successfully determined. In terms of Pst infection, *Acidovorax* sp. leaf 84, *Pedobacter* sp. leaf 194, *Plantibacter* sp. leaf 1 and *Pseudomonas citronellolis* P3B5 showed a striking plant-protective ability in addition to *Sphingomonas* species. Among these strains, only *Plantibacter* sp. leaf 1 belongs to Gram positive strains whilst the others are Gram negative. One reason is that more Gram negative strains (18 strains) were screened than Gram positive strains (9 strains), thereby giving them a higher chance to be more prevalent in the protective strain list. In accordance with this result is that most bioagents again Pst that have been reported so far are Gram negative strains (Innerebner et al., 2011; Morella et al., 2019; Romero et al., 2016). In addition to the feature of Gram positive or negative, these protective strains cover all phyla (Actinobacteria, Bacteroidetes and Proteobacteria) and indicate a low taxa-overlap, which might infer the protective ability of bioagents is not related to bacterial taxa levels. Moreover, these protective strains did not show strong abilities of keeping a lower Pst population than those of axenic plants statistically (p value > 0.99). However, in terms of mean Pst cell numbers, plants that were pre-colonised with *P. citronellolis* P3B5 (Pst = 10^6 cfu/g) or *Acidovorax* sp. leaf 84 (Pst = 10^5 cfu/g) are significantly lower than axenic plants (Pst = 10^9 cfu/g). Since *P. citronellolis* P3B5 and Pst are from the same genus, they might have a high niche-overlap. This may reduce the ability of Pst to

coexist with *P. citronellolis* P3B5 (Remus-Emsermann, Kowalchuk, et al., 2013; Wilson & Lindow, 1994). This is in line with the observation that plants inoculated with *P. koreensis* P19E3 also host low Pst populations (10^7 cfu/g) although only showing intermediate protection. It has been reported that some *Pseudomonas* strains can directly inhibit the growth of Pst as the ability of these strains to produce antimicrobial compounds (e.g. siderophore) has been characterised (Völksch & May, 2001; Wensing et al., 2010). Therefore, for these *Pseudomonas* strains, their protective ability could result from their competitive relationships with Pst. Interestingly, *P. syringae* B728a showed intermediate protective ability while mono-inoculation of *P. syringae* B728a caused adverse effects on Arabidopsis growth (Fig 2.3). Meanwhile, the presence of *P. syringae* B728a did not significantly reduce Pst population (10^8 cfu/g). This excludes the competitive relationships between Pst and *P. syringae* B728a even though antagonistic interactions between Pst and other *P. syringae* strains have been observed (Romero et al., 2016; Völksch & May, 2001).

The average Pst population size from plants pre-colonised with the other protective strains compared with that of axenic plants were not significantly different, which indicates that the plants were more resistant to Pst infection even though the plants hosted a considerable pathogen population. For some non-pathogenic bacteria such as *Bradyrhizobium* sp. leaf 396 and *Variovorax* sp. leaf 220, even though they kept Pst cell numbers lower than those of axenic plants, they did not show protective ability in suppressing disease development. This raises the question of the mechanism of plant protection in addition to microbe-microbe antagonism.

Helfrich et al. (2018) has shown *in vitro* that only the Arabidopsis indiginous bacteria *Sphingomonas* sp. leaf 2 and *Pseudomonas* sp. leaf 58 showed strong inhibitory activities against Pst. By analyzing the bacterial transcriptomes and proteomes of other *Pseudomonads*, Nobori et al. (2020) found large differences at bacterial RNA and protein levels during host infection compared to *in vitro* culture, especially in the early stages of plant-pathogen interaction. It was also found that plant immunity regulated by SA signalling is involved in the suppression of bacterial pathogenesis-related mRNAs and proteins (Nobori et al., 2020). This finding strongly infers that plant hosts are involved in these plant protection activities. Transcriptome study conducted by Vogel et al. revealed that plant hosts can differentially respond to *Sphingomonas melonis* Fr1 and *Methylobacterium extorquens* PA1 (Vogel et al., 2016). Plant host gene expression was hardly affected by the presence of *M. extorquens* PA1 but was significantly changed by *S. melonis* Fr1 (Vogel et al., 2016). Moreover, their study also found defence response gene expression triggered by *S. melonis* Fr1 was partly overlapping with responses to Pst infection (Vogel et al., 2016). Therefore,

the underlying protective mechanisms of the here-tested bacterial strains may be partly explained by activation of plant host immune responses. This led me to investigate host immune responses in the following chapter to investigate whether plant immune system and phytohormone levels are modulated differently between protective strains and nonprotective strains.

2.4.4 Plant-protection against *Botrytis cinerea* by non-pathogenic preemptive colonisation

For *B. cinerea*, all bacterial strains were screened including Pst. Due to time limitations, this experiment was only conducted once. All *Pantoea* strains showed protective ability against *B. cinerea.* Moreover, *P. agglomerans* 299R and *P. vagans* C9-1 kept the plant mortality rate at 0%. Plant phenotype showed that the presence of *Pantoea* strains can significantly reduce the fungal lesion (Fig 2.9). For *P. agglomerans* 299R, the whole genome sequence revealed that the strain does not carry any genes relating to antifungal products (Remus-Emsermann, Kim, et al., 2013). However, it has been reported that other *P. agglomerans* strains (e.g. *P. agglomerans* CPA-2) can be used to control *B. cinerea* infection (Nunes et al., 2002). Genome analysis found the presence of genes in *P. vagans* C9-1 to encode antimicrobial peptide pantocin A and herbicolin I (Kamber et al., 2012; Smits et al., 2019). Three *Sphingomonas* strains (*S.* sp. leaf 17, *S. phyllosphaerae* FA2, *S. melonis* FR1) also exhibited protective ability. However, interestingly, plants pre-colonised with *S*. sp. leaf 34 were even more susceptible to *B. cinerea* infection and the mortality rate was twofold larger than that of axenic plants after 96 hours post infection (Fig 2.8). Two *P. syringae* strains both show a striking protective ability. Their biocontrol activity might be attributed to the production of antifungal metabolites. For instance, genome analysis showed the presence of *PhzF* genes in both Pst and *P. syringae* B728a genomes, which encode for the antifungal product, phenazines (Chin-A-Woeng et al., 2003). In addition, since Pst could produce coronatine to up-regulate JA-related gene expression, this might partially explain that plants with Pst are more resistant to fungal infection as JA signalling response might be enhanced by the presence of Pst. Research has shown that constitutive expression of JA positive upregulators resulted in significantly increased resistance against *B. cinerea* (Moffat et al., 2012). Based on results gathered so far, there are not many clues to extrapolate the possible reasons behind these protections against *B. cinerea* infection. However, the findings of this study open many possibilities that can explain the protective ability of phyllosphere bacteria against *B. cinerea*. For instance, staining technology could be employed such as Trypan blue to observe the suppression of fungal hyphae growth in the

plant leaves (Boedijn, 1956). Moreover, *in vitro* experiments can be conducted to validate their inhibitory interactions.

Chapter 3. Protoplast Assay Development

3.1 Introduction

The *in planta* assay has shown that certain bacterial strains can confer protection against Pst and *B. cinerea* infection. However, this method cannot dissect underlying causal reasons for protection, such as stimulation of defence signalling events in plant host, competitive microbe-microbe interactions or antagonism (Remus-Emsermann, Kowalchuk, et al., 2013; Vogel et al., 2016; Zengerer et al., 2018). In order to provide a higher resolution of molecular mechanisms at the cellular level, a cell-based system employing plant mesophyll protoplasts was constructed in this study. The isolation of plant protoplasts has been used for more than 40 years (Cocking, 1960). Protoplasts are versatile tools to measure plant cellular processes to study plant physiology. Most plant protoplasts are used for transient gene expression studies. For instance, transient gene expression studies have widely been used to elucidate various hormone signalling pathways, protein-protein interactions and single-cell imaging (Birnbaum et al., 2003; Cho et al., 2006; Yanagisawa et al., 2003).

As mentioned in Chapter 1, plant immune responses will be elicited upon detecting microbes. Whereas SA-dependent defence responses are usually employed to combat biotrophic pathogens, JA-dependent defence responses are generally employed to combat necrotrophic pathogens. Therefore, in my project, mesophyll protoplasts of wild-type and two transgenic Arabidopsis lines (pPR1-eYFP-NLS and pVSP1-eYFP-NLS) were isolated. *Pathogenesis-Related Gene 1 (PR1)* and *Vegetative Storage Protein 1 (VSP1)* are conventional makers for SA and JA signalling respectively. Protoplasts isolated from these transgenic Arabidopsis lines express yellow fluorescence when SA- or JA-dependent defences are activated. The experiments in this chapter aimed to test the applicability of this protoplast assay and to optimise the parameters of the assay. Parameters of the plate reader were optimised such as gain, filter channels, etc. Three important signals such as autofluorescence from protoplasts, yellow fluorescence promoted by *PR1/VSP1* expression and mScarlet fluorescence which was used to show bacterial growth. Autofluorescence derived from plant chlorophyll was utilised as a marker to detect living protoplasts (Guadagno et al., 2017). Yellow fluorescence directly revealed the plant immune response as *PR1* and *VSP1* are markers for SA and JA signalling respectively. mScarlet fluorescence was used to measure bacterial growth. The following results will show the feasibility and limitations of the protoplast assay and its optimisation.

3.2 Materials and Methods

3.2.1 Plant material and plant growth conditions

The wild type Arabidopsis was col-0 ecotype. Two transgenic lines of Arabidopsis, pPR1-eYFP-NLS and pVSP1-eYFP-NLS, were kindly provided by Betsuyaku (University of Tsukuba, Tsukuba, Japan). Arabidopsis for protoplast isolation: Plants were cultivated as described by Miebach et al. (Miebach et al. 2020). Briefly, plants used for protoplast isolation were cultivated as follows: 200 µl pipette tips were filled with 60 µl ½ MS agar and then the tip and top of the tips were removed using flame sterilised scissors. The middle part was kept (around 5 mm in length). Afterwards, these middle parts were stabilised onto ½ MS agar in petri dishes. Subsequently, surface-sterilised Arabidopsis seeds were sown onto the tips (1 seed/tip) and the petri dishes were placed in the growth chamber for one week. Then, seedlings which successfully germinated were transferred into a tissue culture box (Magenta vessel GA-7, USA) which contained 100 ml ½ MS agar (4 seedlings/box). Intact seedlings for confocal microscopic check: Arabidopsis seeds were sown onto ½ MS agar in square plates. These square plates were then placed in the plant growth chamber vertically, which allowed the seedlings to grow on the agar surface. The growth conditions are described in 2.1.1.

3.2.2 Mesophyll protoplasts isolation

Leaf protoplasts were prepared following protocols with a minor modification from Yoo et al and Wu et al (F.-H. Wu et al., 2009; Yoo et al., 2007). 10 ml enzyme solution was prepared freshly in advance: To that end 1 ml of 1 M 2-ethanesulfonic acid (MES, Duchefa, Netherlands) (pH 5.7) was mixed with 100 mg cellulase R10 (Duchefa, Netherlands), 25 mg macerozyme R10 (Duchefa, Netherlands) and 200 µl of 1 M KCl. The solution was incubated at 55 °C for 10 minutes and then cooled to room temperature. Afterwards, 100 µl of 1M $CaCl_2$ and 100 µl of 10% (v/v) BSA were added to the solution. After the final volume was adjusted to 10 ml with ddH_2O, the enzyme solution was thoroughly vortexed. The enzyme solution was filter-sterilised through a 0.2 µm syringe filter into a petri dish. The wash solution W5 (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose and 2 mM MES (pH 5.7)) was pre-chilled at 4 °C.

Masking tape (Dixon, New Zealand) and autoclave tape (3M, USA) were UV sterilised in a biological safety hood in advance for 15 minutes. Mature and healthy leaves were cut off

from 5-7 weeks old plants using sterile scissors. Then the leaves were picked immediately with a sterile tweezer and fixed onto the autoclave tape. The epidermal surface of freshly cut leaves was affixed to a strip of autoclave tape and the lower epidermal surface was affixed to a strip of masking tape. Then the masking tape was carefully peeled away from the lower epidermal surface. The remaining parts of the leaves still adhering to autoclave tape were immediately submerged into the enzyme solution in a petri dish. The petri dish was placed on a platform shaker and the leaves were being shaken orbitally at 40 rpm for 60-90 min at room temperature until the solution turned green. Afterwards the solution was transferred into a 50 ml Falcon tube carefully and centrifuged at 100 *g* for 3 min. The supernatant was discarded and the protoplast pellets were washed by 20 ml pre-chilled W5 solution. Centrifugation and washing steps were repeated twice. In the end, the protoplast concentration was examined in a hemocytometer (Neubauer, USA) and adjusted to about 10^5 cells/ml and stored at 4 °C.

3.2.3 Bacterial growth in W5 buffer

The method to subculture bacteria is described in 2.2.2. Bacterial cells were centrifuged at 4000 *g* for 5 min, washed twice with W5 buffer, then cell density of each strain was adjusted to roughly 10^7 cfu/ml. 200 µl of bacterial suspensions of each strain were added into three replicate wells of a 96-well plate.

3.2.4 Positive control test and *Pseudomonas syringae* DC3000 treatment

Transgenic Arabidopsis containing the SA marker, pPR1-eYFP-NLS lines were treated with exogenous SA treatment as positive control. Exogenous SA was diluted in ddH_2O. In a 96-well plate (Corning, USA), 180 µl protoplast solution (concentration: about 8.4×10^4 cells/ml) was mixed with 20 µl SA in each well until final SA concentration was 2 mM, 0.2 mM and 0.02 mM. As SA was dissolved and diluted in ddH_2O. ddH_2O was used as mock treatment. Each treatment had three biological replicates. Pst subculture method was described in 2.2.2. Pst cells were centrifuged at 4000 *g* for 5 min and washed twice with W5 solution and OD_{600nm} was adjusted to 0.45 (equivalent to 2×10^7 cfu/ml, Supplement table 2), 0.22, 0.11, 0.06. In each well, 100 µl protoplast solutions of either pPR1-eYFP-NLS or pVSP1-eYFP-NLS line (concentration: 10^5 cells/ml) were mixed with 100 µl Pst solutions of different concentrations respectively. Each treatment had three biological replicates.

3.2.5 Confocal microscopy and real-time fluorescence detection

Seedlings for the confocal microscopy were seven days old after sowing. pPR1-YFP-NLS seedlings were treated with 0.2 mM SA for three hours. Then seedlings were mounted on a slide for microscopy (Leica SP5, Germany). Fiji/ImageJ was used for image processing. The contrast of images have been improved and background signals have been subtracted. Protoplast suspension of wild type Arabidopsis was detected by sequential scanning. The absorption and emission of protoplasts autofluorescence were measured in a Cary Eclipse Fluorescence Spectrophotometer by a sequential scanning (Agilent Technologies). In terms of real-time fluorescence detection, the 96-well plate was covered with a plastic lid to avoid pressure differences and water evaporation. Then the plate was placed into a plate reader (BMG Labtech, Germany). The measurement setup is described in Table 3.1.

Table 3. 1 Detection parameters in the microtiter plate reader assay

Detected signals	Excitation filter bandpass (nm)	Emission filter bandpass (nm)	Gain	The frequency of plate reads	The diameter detected per well	Temperature
Protoplast autofluorescence	485/14	690/14	2000	160 flashes/well	3 mm	25 °C
Yellow	485/14	520/12				
mScarlet	544/17.5	590/13				

3.2.6 Data analysis

Background signals (W5 buffer) of each biological replicate of each treatment were removed. Then the data was smoothed by built-in function “Fit Spline/LOWESS” (Number of knots: 5) in Graphpad prism 8. After that, the data of each biological replicate was grouped by treatments and for further plots.

3.3 Results

3.3.1 Development of a gnotobiotic protoplast assay

Fresh protoplasts were distributed into a 96-well plate under gnotobiotic conditions and then inoculated with different individual strains simultaneously. The two transgenic Arabidopsis lines pPR1-eYFP-NLS and pVSP1-eYFP-NLS are capable of expressing yellow fluorescence when the marker genes of SA and JA signalling are triggered respectively. The yellow fluorescence along with protoplast autofluorescence and mScarlet fluorescence of bacteria were monitored in the plate reader, when applicable.

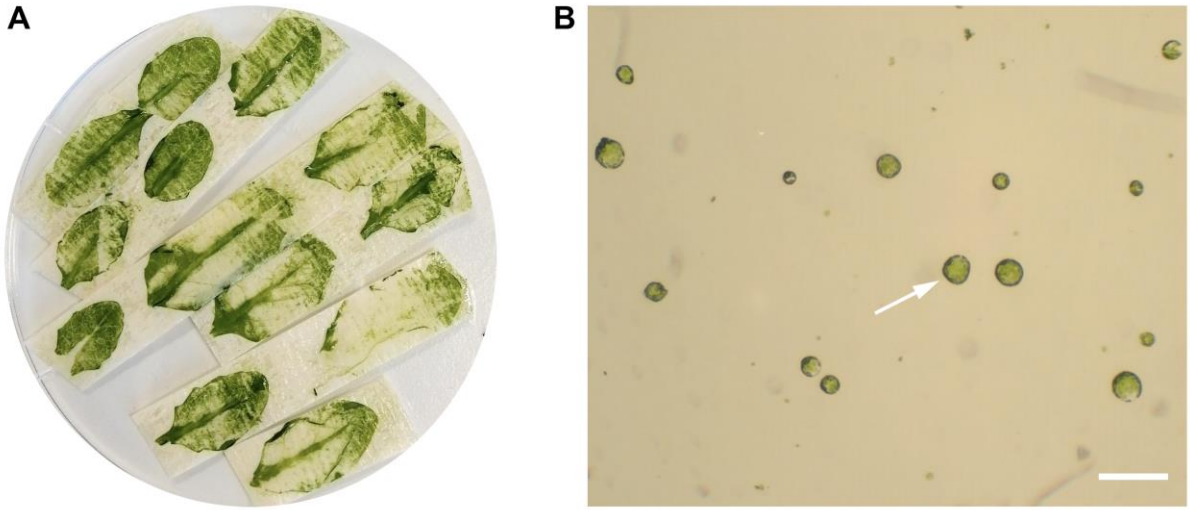


Figure 3. 1 Snapshots of leaves and protoplasts after isolation. (A) Seven-week-old Arabidopsis leaves after 90 min incubation in enzyme solution. Few leaves have been completely digested. (B) Most protoplasts were intact and viable. Pointed out by arrow. The protoplast yield was about 2 x 10^6 cells per gram of fresh leaves. Scale bar = 100 µm.

Bacteria were incubated in W5 buffer to test whether W5 buffer impacts on bacterial growth and whether bacterial autofluorescence impacts on other fluorescence channels. All strains exhibited autofluorescence in the range of yellow fluorescence from protoplasts which means bacterial autofluorescence can impact the YFP signals (Supplement Fig 2A). Most mScarlet expressing bacterial strains showed a stable or increasing population except for Pst which exhibited a decrease in mScarlet fluorescence in W5 buffer (Supplement Fig 2B).

3.3.2 Confocal microscopy of wild-type plant protoplasts and transgenic seedlings

Fresh protoplasts isolated from wild-type Arabidopsis were examined under confocal microscopy (Fig 3.2). A lambda scan showed that protoplasts exhibited autofluorescence from ~645 to 780 nm with a peak emission at around 670 to 690 nm (Fig 3.2B). This result was corroborated by a UV spectrum analysis where protoplasts showed the highest fluorescence around 690 nm (Fig 3.2C).

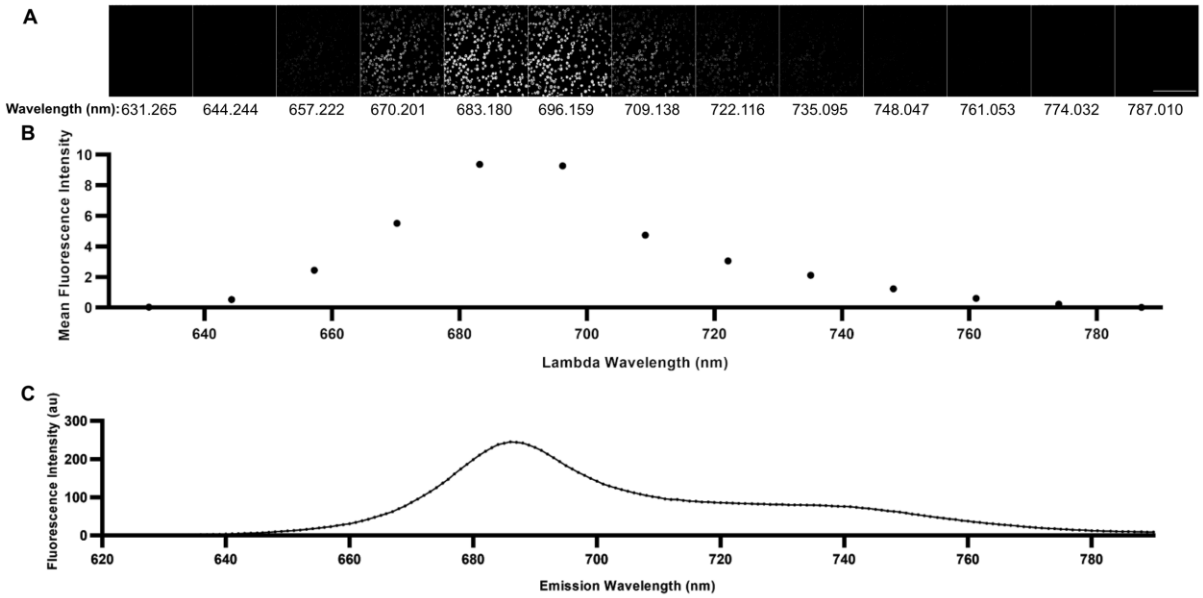


Figure 3. 2 Emission spectrum of autofluorescence of wild type protoplasts. (A-B) Lambda scan of protoplasts of wild-type Arabidopsis. (A) The mean relative fluorescence intensity of raw pictures was measured. Scale bar = 100 µm. (B) Each point in the scatter plot corresponds to the picture above in A. (C) Protoplast autofluorescence emission under UV spectrum. The excitation wavelength measured by a fluorescence spectrum was set at 481 nm.

Intact seedlings were checked by confocal microscopy to determine the *PR1* promoter activity and quality assurance of regenerated seeds. Compared to mock treatment (Fig 3.3A), 0.2 mM SA strongly induced eYFP expression in the whole seedling including cotyledons and roots (Fig 3.3B, C, D).

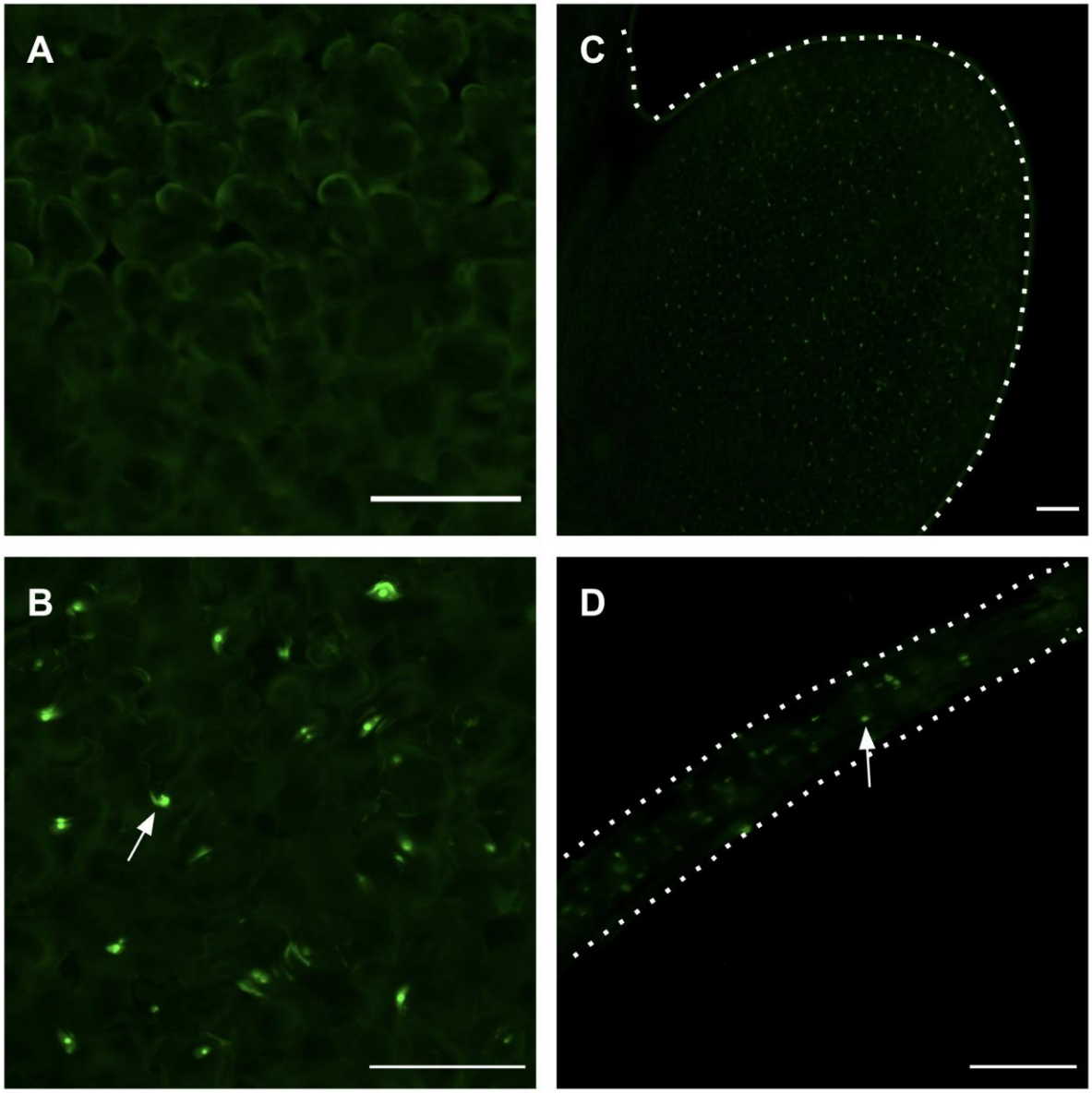


Figure 3. 3 Representative images of nuclear-localized eYFP signals in seven-day-old seedlings. eYFP expressing nuclei are indicated by arrows. (A) eYFP expression in mock treated seedlings. (B) eYFP expression in 0.2 mM SA-treated seedlings. (C-D).eYFP expression of 0.2 mM SA treated seedlings in cotyledon (surrounded by dashed line) (C) and roots (surrounded by dashed line) (D). Scale bars = 100 µm

3.3.3 Positive control optimization

To determine the amplitude of the eYFP expression of transgenic Arabidopsis protoplasts, exogenous SA was applied. An experiment was conducted to determine the optimal SA concentration that results in a SA signalling pathway response as determined by a strong eYFP signals. It is noteworthy that protoplasts without SA treatment still showed a slight increase in eYFP fluorescence (Fig 3.4A). With the increase of exogenous SA concentration (from 0 to 0.2 mM), protoplast responded similarly with an increase in eYFP fluorescence, peaking at around 11 hours (Fig 3.4A). However, eYFP fluorescence of protoplasts treated

with 2 mM SA only increased slightly in the first 5 hours before remaining at a relatively low fluorescence of 6000 au. Microscopy of protoplasts after 17 hours post treatment confirmed that protoplasts treated with 2 mM SA did not show any nuclear-localized signals, suggesting they were probably dysfunctional (Fig 3.4E) whilst protoplasts from other treatments showed nuclear-localized YFP signals (Fig 3.4B-D).

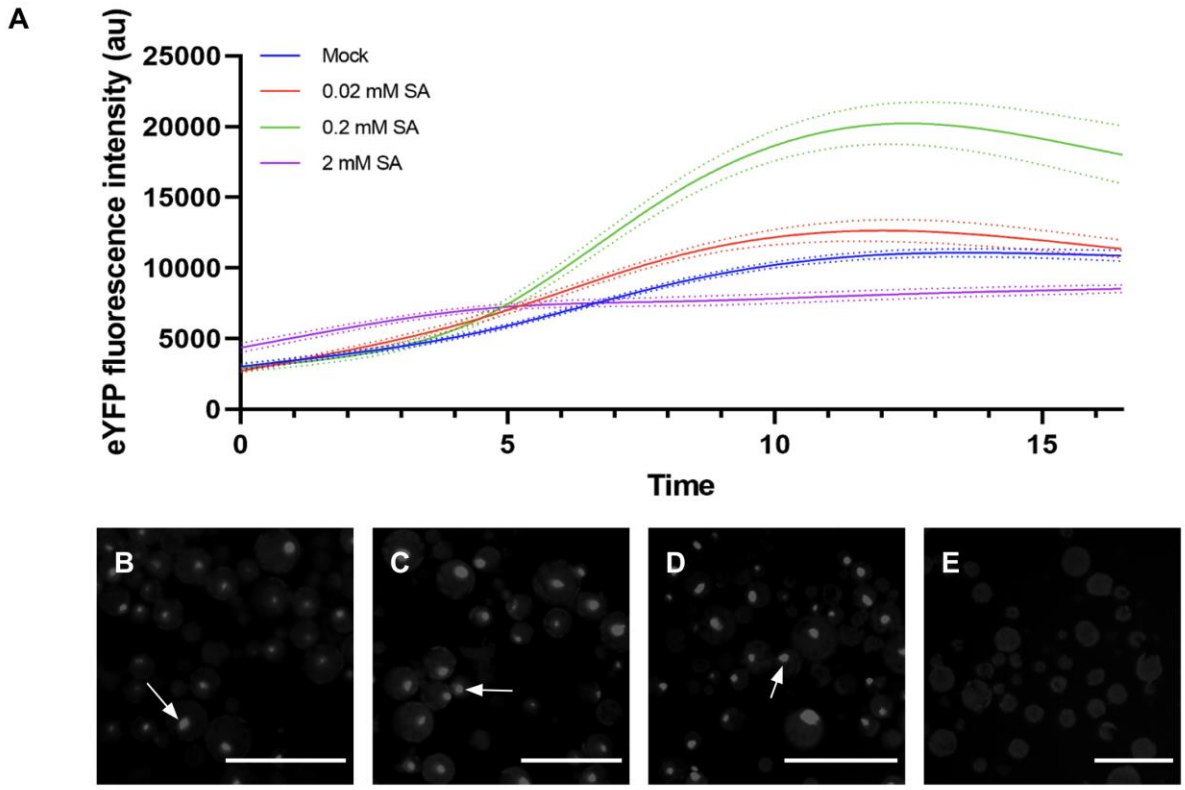


Figure 3. 4 Optimizing SA concentration. (A) PR1 promoter dynamics upon treatment with SA or mock. Dashed lines show standard deviation. (B-E) Nuclear-localized eYFP expression of protoplasts 17 hours post treatment is pointed out by arrows. Exposure time was set to 1000 ms. (B) Mock treatment, (C) 0.02 mM SA treatment, (D) 0.2 mM SA treatment, (E) 2 mM SA treatment. Scale bar = 100 µm.

In a subsequent experiment, SA was used at a concentration of 0.2 mM. In a longer term observation, it was apparent that the yellow fluorescence intensity drastically soared to over 25000 au after 36 hours (Fig 3.5A). At the same time, protoplast autofluorescence increased from 28 hours to 35 hours but then dropped to lower than 15000 au (FIG 3.5A). A microscopical check showed that protoplasts treated with SA after 48 hours contained more condensed and seemingly shriveled protoplasts compared to mock-treated protoplasts (Fig 3.5B-C).

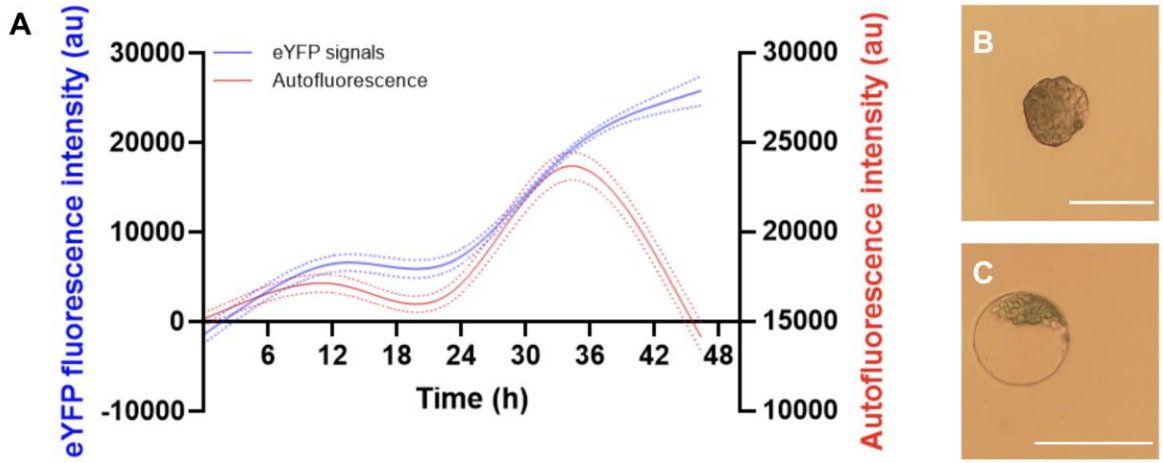


Figure 3. 5 Optimizing treatment time. (A) PR1 promoter dynamics and protoplast autofluorescence with 0.2 mM SA treatment for 48 hours. (B) Protoplasts treated by 0.2 mM SA after 48 h. Scale bar = 50 µm. (C) Mock treated protoplasts after 48 h. Scale bar = 50 µm.

3.3.4 Optimizing bacterial treatment

In order to determine the potential bacterial behavior in the presence of protoplasts and whether different transgenic protoplasts have different impact on bacterial growth, the model biotrophic pathogen Pst was cultured in W5 solution containing protoplasts and measured in the plate reader. For this experiment, the protoplast density was adjusted to about 10^4 cells per well. Since the red fluorescence intensity of mScarlet expressing Pst is directly correlated with the optical density of Pst (Fig 3.6A), it is possible to infer the bacterial population density by the corresponding mScarlet fluorescence intensity. Two-fold serial dilution of a 10^6 CFU/well Pst suspension was tested in order to find the optimal concentration of bacterial cell suspension (Fig 3.6B). Low densities of Pst (from 1.25 x 10^5 to 5 x 10^5 cfu/well) maintained the same fluorescence intensity for the duration of the experiment while mScarlet intensity of Pst with 10^6 cfu/well declined from 30000 au to 8000 au.

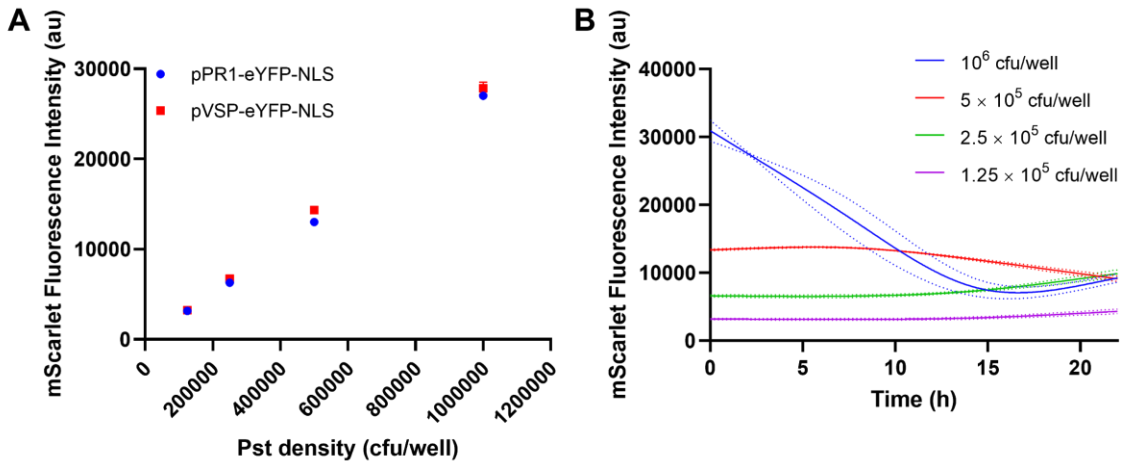


Figure 3. 6 Pst behaviour in the presence of protoplasts. (A) The incremental relationship of Pst optical density with mScarlet fluorescence intensity at time point 0. Blue dots represent Pst intensity in the presence of pPR1-YFP-NLS protoplasts, red dots represent Pst intensity in the presence of pVSP1-eYFP-NLS protoplasts. Error bars indicate the standard deviation of three independent replicates. (B) mScarlet fluorescence of Pst as a proxy for population development over time. Data of pPR1-eYFP-NLS and pVSP1-eYFP-NLS protoplasts was combined. Dashed lines show standard deviation.

3.4 Discussion

The protoplast isolation method was based on epidermal peeling rather than traditional slicing and vacuum (Hansen & van Ooijen, 2016; F.-H. Wu et al., 2009). This peeling method is gentler and yields a higher abundance of protoplasts (Barnes et al., 2019; F.-H. Wu et al., 2009). More importantly, it has significantly reduced isolation time, which minimises the risk of protoplast being exposed to abiotic stress. Another advantage of the epidermal peeling technique is that it is easier to work aseptically compared to the problems to maintain sterile conditions in a vacuum desiccator.

Since autofluorescence derived from plant chlorophyll can be employed to reflect the health status of protoplasts, the emission wavelength to detect protoplast autofluorescence was determined (Guadagno et al., 2017). In the end, the emission wavelength to measure protoplast fluorescence, eYFP fluorescence and mScarlet protein signals did not overlap (Table 3.1). In the next step, pPR1-eYFP-NLS protoplasts were tested with different SA concentrations and different treating times to determine the optimal experimental setting. In these experiments, protoplasts treated with 2 mM SA only had a short and mild response in the first five hours before remaining at 6000 au. One possible explanation is that protoplasts responded to 2 mM SA with a hyper-sensitive response. Microscopy analysis confirmed that protoplasts treated with 2 mM SA were unable to produce eYFP signals (Fig 3.4E). Mock treatment (ddH_2O) and 0.02 mM SA treatment induced slight SA response. Here, osmotic

stress might play the main role for the observed SA response since W5 buffer concentration was slightly reduced by adding ddH_2O or SA solution. The strongest *PR1* expression was observed upon 0.2 mM SA treatment.Therefore, this concentration was subsequently used as a positive control. In order to avoid the loss of protoplast function, the measuring time was reduced to 24 hours in the subsequent formal experiments.

For bacterial treatments, Pst was used to determine the potential bacterial behavior in the presence of protoplasts. Only the mScarlet signals of the highest Pst cell concentration dropped down drastically. On the other hand, mScarlet intensity of other concentrations remained stable. I assume that low pathogen concentrations would not produce enough MAMPs to induce strong plant immune responses. Thus, the bacterial cell density (10^7 cfu/ml) mixed with 10^5 protoplast cell/ml was applied in subsequent experiments in order to trigger plant immune responses. Moreover, since all bacterial strains exhibit bacterial autofluorescence that impacts the YFP signal channel and Pst behavior was almost the same in the presence of pPR1-eYFP-NLS or pVSP1-eYFP-NLS protoplasts, bacteria were co-cultured with transgenic protoplasts or WT protoplasts respectively in the later experiments of chapter 4. Bacterial autofluorescence can be removed by subtracting values of WT protoplasts treated with the exact same bacteria from total values of transgenic protoplasts to determine theeYFP signals related to changes in *PR1* expression.

pVSP1-eYFP-NLS protoplasts were also tested by different concentrations of ethanol or methyl jasmonate. However, there were no measurable signals detected in the plate reader. Microscopicy showed only a few protoplasts produced weak eYFP signals. Due to time limitations, it was not possible to establish a microtiter plate assay for pVSP1-eYFP-NLS protoplasts. In the subsequent experiments of chapter 4, only pPR1-eYFP-NLS protoplasts were used. Overall, my protoplast assay provides the opportunity to measure a dynamic plant SA-dependent defence response along with bacterial behavior. Moreover, up to 13 bacterial strains can be tested simultaneously in one experiment. These advantages can help us figure out how plant phytohormone levels are modulated in the presence of different bacteria in a high throughput fashion and extend our understanding of bacterial plant-protection.

Chapter 4. Plant immune response to various bacterial strains

4.1 Introduction

Chapter 2 provides evidence that some bacterial strains can provide Arabidopsis with some protection against Pst or *B. cinerea* infections. However, the underlying protective mechanisms cannot be conclusively dissected by plant phenotype screening and bacterial population levels. For instance, *Sphingomonas* strains did not suppress the Pst population but showed a striking protective ability whilst plants inoculated with *Variovorax* sp. leaf 220 host a relatively small Pst population but still showed susceptible disease. This raises the question whether the plant host's reaction is involved in the protective effect. A number of studies have demonstrated that the plant innate immune system is required for the defence against plant pathogens (Glazebrook, 2005; Nobori et al., 2020; Vlot et al., 2009). Thus, I hypothesise that the presence of protective bacteria can trigger the plant immune response. I hypothesise that protective bacteria against Pst induce strong SA signalling response and protective bacteria against *B. cinerea* and non-protective bacteria will not have impact on SA signalling. I hypothesise that the plant responds by going into a higher state of alert or defence mode, which makes it more resistant when encountering pathogens later.

In order to demonstrate the role of plant immune response in bacterial protection, a protoplast assay has been established as described in chapter 3. The protoplast assay provides a time resolved analysis and generates high resolution data of the activity of the plant defence-related promoter *PR1* and bacterial behaviour in response to the plant defence *in situ*. Fluorescence microscopy was employed to validate the fluorescence signals and the viability of protoplasts at the end time point. The hypothesis that SA response pathways can be activated by protective strains and non-protective bacterial strains might not have lasting effects on the plant immune system was tested. Therebye, the protoplast assay leads to high resolution understanding of plant-bacteria interactions and helps us gain mechanistic insights into bacterial biocontrol activities.

4.2 Materials and Methods

4.2.1 Plant material and plant growth conditions

See 3.2.1

4.2.2 Mesophyll protoplasts isolation

See 3.2.2

4.2.3 Non-pathogen and pathogen treatment

Bacteria were cultured as described in 2.2.2. Bacterial cells were centrifuged at 4000 *g* for 5 min and washed twice with 80% (v/v) W5 buffer. Then the cell density of each bacterial strain was adjusted to roughly 2 x 10^7 cfu/ml with 80% W5 buffer according to Supplement table 2. 100 µl protoplast solution and 100 µl bacterial suspension of each strain were mixed in triplicates into wells of a 96-well plate to a volume of 200 µl in each well (protoplast: 25000 cells/well, bacteria: 10^6 cfu/well). 80% W5 buffer was applied as mock treatment.

4.2.4 Real-time fluorescence measurement

See 3.2.5

4.2.4 Fluorescence microscopy

After measurement in the plate reader, protoplasts were immediately observed with an Olympus IX70 fluorescence microscope (Olympus, Japan) at 20x magnification (LCPlanFi 20X/0.04, Japan) with Leica filter set U-MWU (excitation bandpass 330-385 nm, dichroic mirror 400 nm, emission long pass filter 420nm), an Axiocam MRc5 and software AxioVision 4 (version 4.8.2.0) for the detection of eYFP signals. eYFP was recorded with three different exposure times (10 ms, 50 ms and 200 ms) to cover the heterogeneity of the fluorescently tagged nuclei and avoid over and underexposure. Bright field images were exposed for 6 ms.

4.2.4 Data analysis

Background signals (W5 buffer) of each biological replicate of each treatment were subtracted. Then data was smoothed by built-in function “Fit Spline/LOWESS” (Number of knots: 5) in Graphpad prism 8. After that, the data of each biological replicate was grouped

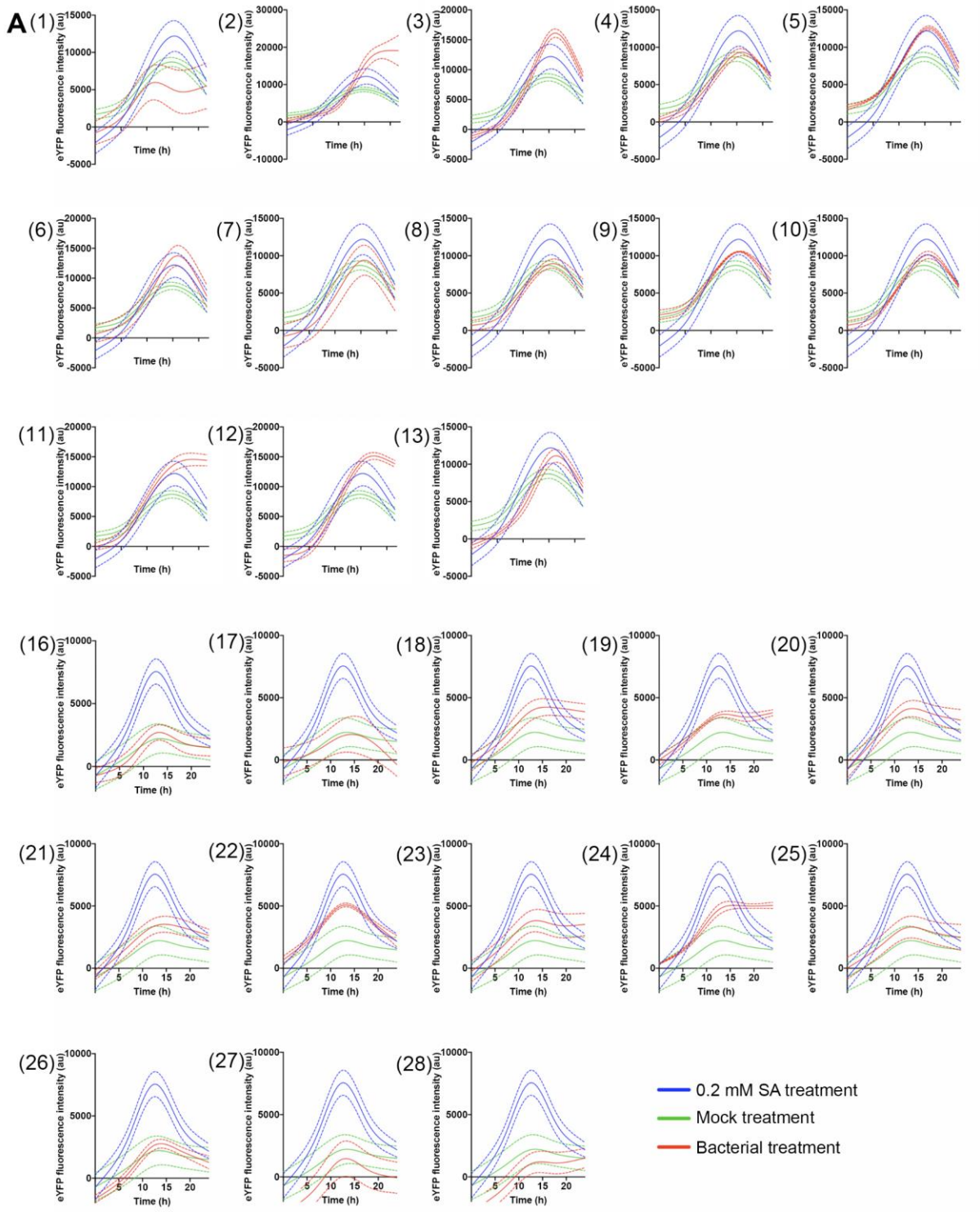
by treatments to plot curves. The overall fluorescence intensity of each picture at the experiment end point was measured by ImageJ.

4.3 Results

As shown in Fig 4.1A and Supplement Fig 3, *Pseudomonas syringae* B728a, *Pseudomonas citronellolis* P3B5, *Pantoea agglomerans* 299R, *Sphingomonas* sp. leaf 17 and *Sphingomonas phyllosphaerae* FA2 strongly induced *PR1* expression which was even stronger than upon treatment with 0.2 mM SA which served as a positive control. *Rhodococcus* sp. leaf 225 and *Rathayibacter* sp. leaf 296 also significantly up-regulated *PR1* promoter activity by more than two fold compared to mock treatment (Supplement Fig 3). The impact of Pst, *Pedobacter* sp. leaf 194 and *Erwinia amylovora* CFBP1430S on *PR1* expression was weaker than mock treatment, however, the protoplasts showed a small up-regulation over time. Other bacterial strains also had the capacity to induce *PR1* promoter activities which were slightly stronger than those of mock treatment. For most bacterial strains, the strongest *PR1* promoter activities were observed after approximately 15 hours (Fig 4.1A, Supplement Fig 3). It is interesting to note that protoplasts that were mock treated or treated with 0.2 mM SA also produced the strongest eYFP signals after approximately 15 hours (Fig 4.1A). However, the *PR1* promoter activity induced by *Agreia* sp. leaf 335, *Pedobacter* sp. leaf 194, *Pseudomonas syringae* B728a and *Pantoea vagans* C9-1 did not reach the peak at the end time point (Fig 4.1A). Moreover, from 20 hours onwards, the eYFP fluorescence intensity of *Pseudomonas syringae* B728a treatment became more variable (Fig 4.1A). A fluorescence microscopical check confirmed that protoplasts treated with some bacteria or SA contained more eYFP expressing protoplasts and the overall images were brighter (Fig 4.2B). eYFP signal intensity had a much larger standard deviation when protoplasts treated with *Pseudomonas syringae* B728a treatment at the end time point from the data of the microscopy (Fig 4.2).

In terms of bacterial behavior in the presence of protoplasts, only *Pseudomonas citronellolis* P3B5 and *Pseudomonas syringae* DC3000 showed a decreasing population whilst other mScarlet tagged strains showed an increase in fluorescence, indicating population growth (Supplement Fig 4). Furthermore, two *Methylobacteria* and a *Methylorubrum* strain exhibited an increasingly varying growth during the experiment (Supplement Fig 4). *Acidovorax* sp. leaf 84 maintained a relatively stable population in the first 15 hours then significantly proliferated the population (Supplement Fig 4). *Pantoea agglomerans* 299R increased in fluorescence intensity until 10 hours after incubation (Supplement Fig 4). All Sphingomonads exhibited time-related growth (Supplement Fig 4). Only the growth curve of ten strains were

determined as only bacteria tagged with constitutively expressed mScarlet proteins can be monitored for their growth in the protoplast assay. This data gave us a hint about how bacteria respond to plant SA signalling responses.



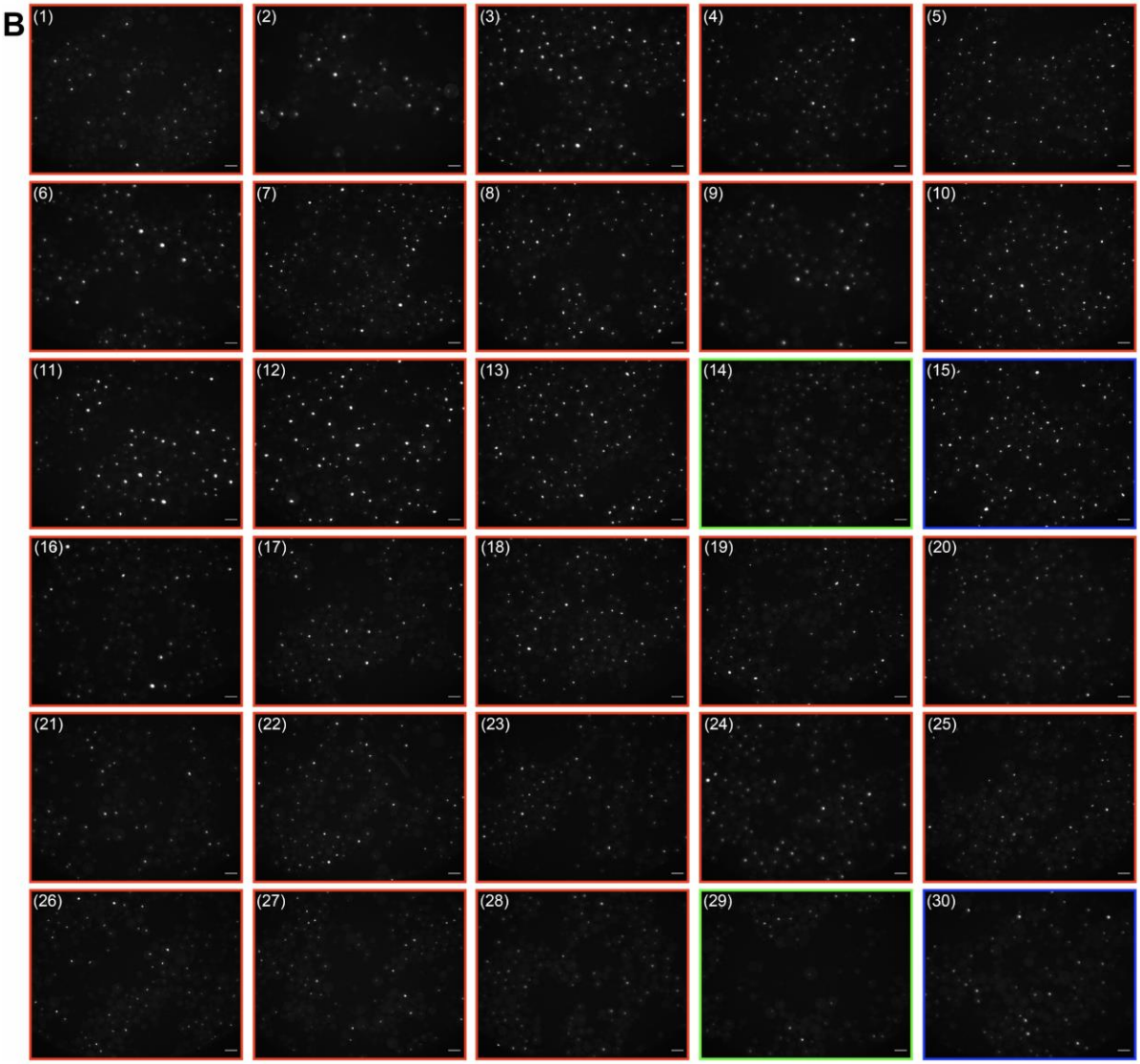


Figure 4. 1 Time-series PR1 promoter activities and the end point microscopy. Numbers represented different treatments. (1) Pseudomonas syringae DC3000, (2) Pseudomonas syringae B728a, (3) Pseudomonas citronellolis P3B5, (4) Sphingomonas melonis FR1, (5) Sphingomonas phyllosphaerae FA2, (6) Sphingomonas sp. leaf 17, (7) Sphingomonas sp. leaf 357, (8) Methylobacterium sp. leaf 85, (9) Methylobacterium radiotolerans 0-1, (10) Methylorubrum sp. leaf 92, (11) Pantoea vagans C9-1, (12) Pantoea agglomerans 299R, (13) Acidovorax sp. leaf 84, (14) Mock treatment, (15) 0.2 mM SA ,(16) Pseudomonas koreensis P19E3, (17) Erwinia amylovora CFBP1430S, (18) Variovorax sp. leaf 220, (19) Agreia sp. leaf 335, (20) Microbacterium sp. leaf 320, (21) Microbacterium sp. leaf 347, (22) Rathayibacter sp. leaf 296, (23) Arthrobacter sp. leaf 145, (24) Rhodococcus sp. leaf 225, (25) Aeromicrobium sp. leaf 245, (26) Williamsia sp. leaf 354, (27) Plantibacter sp. leaf 1, (28) Pedobacter sp. leaf 194, (29) Mock treatment, (30) 0.2 mM SA. (A) Time-series PR1 promoter dynamics in the presence of different bacterial strains. 80% W5 buffer was applied as mock treatment. Dashed lines show standard deviation. (B) Nuclear-localized eYFP expression of protoplasts at the experiment end point (~22 hours post treatment). Pictures are representatives from one of three replicates. Exposure time was set at 200 ms. Scale bar = 100 µm.

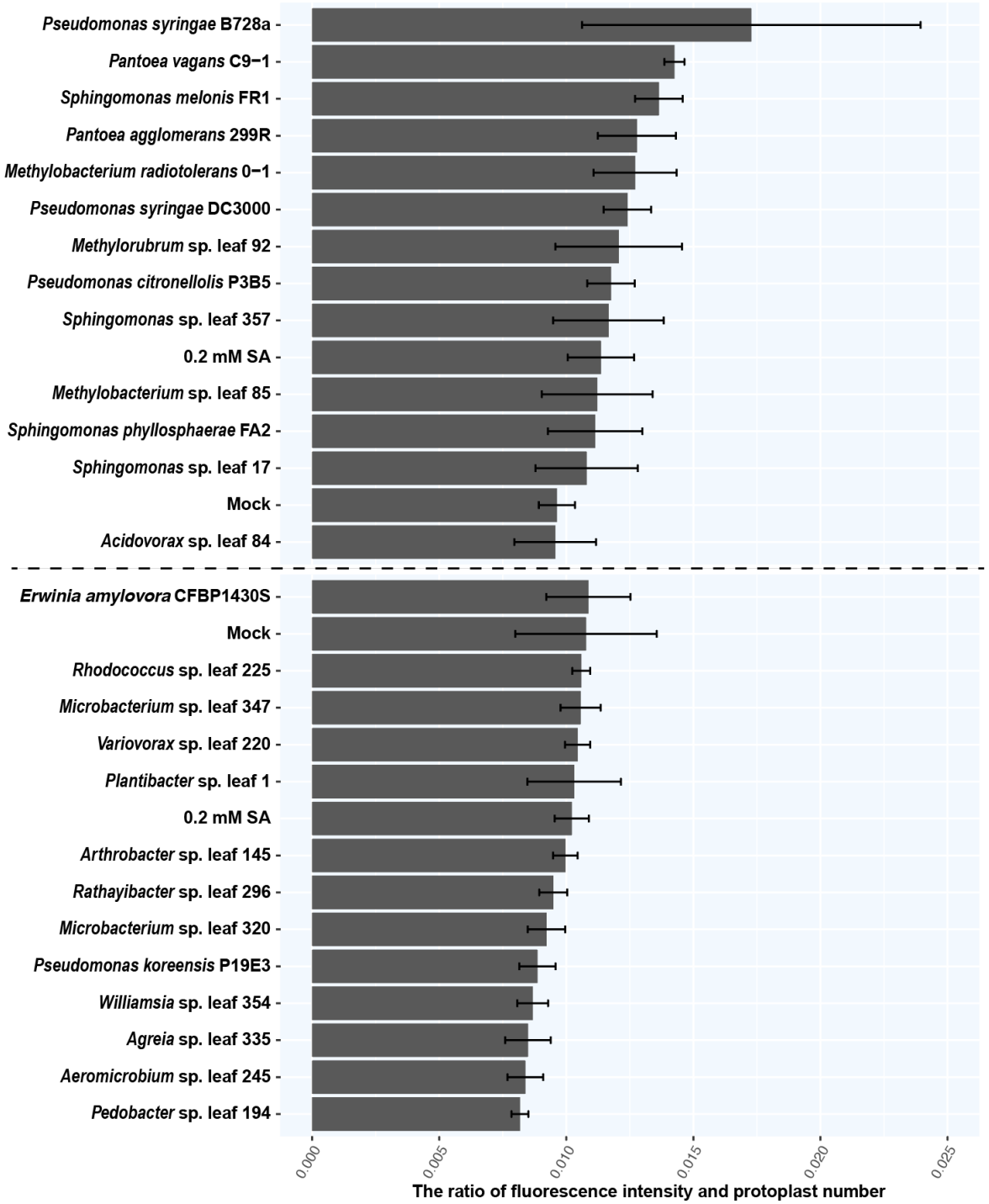


Figure 4. 2 The ratio of eYFP signals and viable protoplast number in the end point microscopic screenshots. The overall fluorescence intensity of each picture was measured using imageJ. The exposure time for these pictures was 10 ms. Number of viable protoplasts in each picture was counted manually. Two experiments were separated by a dashed line.

4.4 Discussion

Plants largely depend on the innate immune system to prevent pathogen infection (Jones & Dangl, 2006). As mentioned in 1.2, SA-mediated mechanisms play an important role in defence against biotrophic pathogens (Glazebrook, 2005). Recognition of potential invaders is crucial to respond in an appropriate manner (Legein et al., 2020). A number of studies reported that non-pathogenic bacteria can also modulate plant immune responses (Tyagi et al., 2018; Vogel et al., 2016). Therefore, it is likely that protective bacterial strains impact on the plant immune system to inhibit pathogens indirectly in addition to direct microbe-microbe antagonistic interactions (Hacquard et al., 2017; Hanemian et al., 2013). In this chapter, I investigated how bacteria modulate the SA signalling of plants and bacterial behavior in response to plant immune responses in a model system. The results show that *Pseudomonas syringae* B728a, *Pseudomonas citronellolis* P3B5, *Pantoea agglomerans* 299R, *Pantoea vagans* C9-1, *Sphingomonas* sp. leaf 17, *Sphingomonas phyllosphaerae* FA2, *Rhodococcus* sp. leaf 225 and *Rathayibacter* sp. leaf 296 strongly induced *PR1* expression while Pst, *Pedobacter* sp. leaf 194 and *Erwinia amylovora* CFBP1430S showed a weaker up-regulation than the mock treatment (Fig 4.1A, Supplement Fig 3). Among these bacterial strains, *S.* sp. leaf 17, *S. phyllosphaerae* FA2, *Pedobacter* sp. leaf 194 and *P. citronellolis* P3B5 are protective strains against Pst. Pst, *P. syringae* B728a, *P. agglomerans* 299R, *S. phyllosphaerae* FA2 and *R.* sp. leaf 225 are protective strains against *B. cinerea*.

In terms of bacterial behaviours, all *Sphingomonas* strains grew in presence of protoplasts as measured by increasing red fluorescence intensity as a proxy for bacterial abundance even though *S.* sp. leaf 17 and *S. phyllosphaerae* FA2 can induce strong SA responses (Fig 4.1A, Supplement Fig 3, Supplement Fig 4). On the other hand, *S.* sp. leaf 357 and *S. melonis* FR1 showed similar SA induction compared to the mock treatment. This shows that the modulation of plant immune responses to *Sphingomonads* is species specific. Vogel et al. showed PR proteins and positive regulators of SA signalling induced significantly by *S. melonis* FR1 after two days inoculation (Vogel et al., 2016), which suggests the plants might need longer time to induce immune response to *S. melonis* FR1. Pst showed a decreasing population similar to that in W5 buffer (Supplement Fig 2B, Supplement Fig 3). Therefore, the suppressing population of Pst is probably a result of the effect of W5 buffer rather than plant SA response. Beyond that, the weaker *PR1* promoter activities suggested that Pst might up-regulate JA signalling to antagonise SA signalling (Büttner, 2016; Vogel et al., 2016). *P. citronellolis* P3B5 showed a decline in population density and it induced the second strongest *PR1* promoter activation (Fig 4.1A, Supplement Fig 4). *In planta* observations showed that the population size of *P. citronellolis* P3B5 was not suppressed in

the phyllosphere (Supplement table 3). This infers that bacteria might behave differently *in planta* and in the protoplast suspension. *P. agglomerans* 299R and *P. vagans* C9-1 are two protective strains against *B. cinerea*, in other words, they might induce strong JA response and weak SA response to inhibit *B. cinerea* growth indirectly. However, they both showed strong ability to trigger SA signalling in my experiment (Fig 4.1A, Supplement Fig 3). It has been reported *Alternaria* sp. (a necrotroph) could induce both JA and SA signalling in *Chrysanthemum morifolium* (Zhao et al., 2020). I hypothesise that SA signalling also plays a role in plant protection by *Pantoea* against *B. cinerea.* as *P. agglomerans* 299R, based on its genome sequence, has not been described to possess antifungal gene products (Remus-Emsermann, Kim, et al., 2013). In the study of Vogel et al., *Methylobacterium extorquens* PA1 hardly caused transcriptional reprogramming of the plant host (Vogel et al., 2016). Here, in my study, all Methylobacteria and *Methylorubrum* spp. exhibited a weak impact on SA signalling similar to mock treatment, which is in line with the presence of Methylobacteria being ignored by plant hosts.

It is interesting that the eYFP intensity of most bacterial treatments and 0.2 mM SA treatment peaked at about 15 hours (Fig 4.1A). Some studies stated that the induction of defence genes could be transient in PTI but prolonged and long-lasting in ETI (Tsuda & Katagiri, 2010; Vogel et al., 2016). In this case, the SA response in my experiments possibly resulted from MAMPs recognition and thereby PTI. Even though most protoplasts still appeared intact at the end of the experiment, some of them did not produce eYFP signals. In other words the fluorescence intensity of protoplasts was not uniform, but rather heterogeneous (Fig 4.1B). It is worth mentioning that the eYFP fluorescence intensity of mock treatment and 0.2 mM SA treatment was comparable in two independent experiments. The intensity of the first experiment was generally higher than that of the second experiment. Also, the fold change of 0.2 mM SA treatment compared to mock treatment was less than 1.5 in the first experiment whilst it was more than threefold in the second experiment (Supplement Fig 3). The differences between the two experiments might be due to different ages of the used plant material as the age of Arabidopsis plants and leaves were not exactly the same. It has been reported young leaves can induce stronger PR1 promoter activities than old leaves (Berens et al., 2019). The differences in plant age were due to the impact of the 2020 Covid-19 lockdown that destroyed parts of my experiments and have put me under considerable time pressure.

In conclusion, different bacteria induce distinct plant SA responses. These differences may be the result of different MAMPs produced by different bacterial strains (Aslam et al., 2009; Gust et al., 2007). Another possibility is that bacteria may interfere with the immune

responses of plants with different strategies. As mentioned above, Pst could up-regulate JA signalling to suppress SA-mediated defence (Büttner, 2016; Vogel et al., 2016). Also, it has been reported that Pst can inhibit plant immune responses by disturbing the perception of 3-hydroxy fatty acids which are MAMPs widely found in Gram negative bacteria (Luo et al., 2020). Therefore, it is possible for non-pathogens to take similar strategies to suppress plant defence immune responses to enhance their adaptation ability. In terms of potential plant-microbe interactions suggested by this study, for *S.* sp. leaf 17, *S. phyllosphaerae* FA2 and *P. citronellolis* P3B5, it is likely that the plant immune system participates in their protection against Pst as they can strongly trigger plant SA signalling. However, for *P. syringae* B728a, *P. agglomerans* 299R, *P. vagans* C9-1, *S. phyllosphaerae* FA2 and *R.* sp. leaf 225, these protective strains against *B. cinerea* also induced SA response. As JA signalling is required for combatting necrotrophic pathogens and due to the antagonistic relationship between SA and JA signalling, it will be interesting to determine whether JA signalling would be induced or not in response to these bacteria in the future and thus uncover the role of SA and JA signalling in defence against *B. cinerea*.

Chapter 5. Conclusion and future work

5.1 Thesis summary and conclusion

In light of public concerns over the use of pesticides and antibiotics in plant protection, it is inevitable to broaden our knowledge about viable biocontrol agents. This study successfully screened a set of bacterial strains which can help Arabidopsis combat the adverse effect of Pst or *B. cinerea* infection. A novel protoplast assay was established to determine the role of plant immune responses in microbial biocontrol activity.

The *in planta* assay revealed a number of strains that had a fully protective or intermediate protective effect against Pst infection (Table 2.6). It has been reported that plant disease severity negatively correlates with pathogen population size (Innerebner et al., 2011; Whalen et al., 1991). Innerebner et al. (2011) showed that plants inoculated with *Sphingomonas* strains host 10^{10} cfu/g Pst which is lower than 10^{8} cfu/g on not *Sphingomonas* inoculated plants. The protective strain *Sphingomonas melonis* FR1 was shown to induce the expression of PR proteins and positive regulators of SA signalling (Vogel et al., 2016). Therefore, I hypothesized that protective strains induce SA responses to confer the plants protection and/or suppress Pst proliferation. However, in my study here inoculation with some protective strains including three *Sphingomonas* strains did not affect Pst population size (light grey and blue background, Table 5.1). In addition, these bacteria did not induce strong SA responses in the protoplast assay (light grey background, Table 5.1). The underlying mechanisms of their protection cannot be fully explained with the data presented in this study. Since stomatal cells play an important role in defence against pathogen infection (Zeng et al., 2010), one possibility might be that these bacteria positively regulate stomatal closure to keep Pst out of plant mesophyll cells. In such case plants would not be attacked severely even under a relatively high population density of Pst. Since the protoplast assay only determined the SA response within the first 25 hours after treatment, a potential role of plant immune responses cannot be fully excluded as SA responses to these bacteria might be induced at a later time. Further, other immune networks might be induced. The hypothesis that non-pathogens can influence plant immune responses to shape Pst population seems to be the case for *P. citronellolis* P3B5. *P. citronellolis* P3B5 kept 10^{6} cfu/g Pst which is 1000 times lower than that of axenic plants and it strongly induced SA responses. For strains *S.* sp. leaf 17 and *S. phyllosphaerae* FA2, *Rathayibacter sp. leaf 296, Pantoea vagans C9-1 and Pseudomonas syringae* B728a, they induced strong SA responses but did not reduce the Pst population. It is likely that plant immune responses are

mediated and pre-activated by these bacteria, thus plants are more resilient to a relatively high population of Pst. On the other hand, SA responses are not significantly induced by *Acidovorax* sp. leaf 84, *Microbacterium* sp. leaf 320 and *Pseudomonas koreensis* P19E3 but Pst cell numbers were reduced by these strains. It is likely that these strains have antagonistic relationships with Pst and diminish the proliferation of Pst via direct or indirect microbe-microbe interactions without the indirect involvement of the plant immune systems. It is interesting that some strains reduced Pst population or induced strong SA responses, but did not show a plant-protective effect. It might be the case that the Pst pathogenicity would be enhanced with the presence of these non-pathogenic bacteria. For those non-protective but SA signalling-induced bacteria, this questions the evitable role of SA signalling in defence against biotrophic pathogen and requires more study on the underlying mechanisms of SA-mediated defence.

Arthrobacter sp. leaf 145, *Pseudomonas syringae* DC3000, *Pseudomonas syringa*e B728a, *Pantoea vagans* PW, *Pantoea vagans* C9-1, *Pantoea agglomerans* 299R, *Rhodococcus* sp. leaf 225, *Sphingomonas* sp. leaf 17, *Sphingomonas phyllosphaerae* FA2 and *Sphingomonas melonis* FR1 were showing a plant-protective effect against *B. cinerea*. Due to time limitations, only plant mortality rate was determined for this experiment. Also, JA responses were not determined for these strains. Interestingly, as shown in 2.3.1, Pst and *P. syringae* B728a exhibited adverse effects on Arabidopsis growth. However, they can help plants reduce mortality rate when encountering *B. cinerea* infection. Protoplast assay shows Pst treatment could suppress SA responses while *P. syringae* B728a strongly induced SA responses. This suggests two *P. syringae* strains use different strategies to employ plant immune responses to confer their protection. Guo et al (2019) showed that infection of Pst or pre-treatment of flagellin enhanced Arabidopsis resistance to *B. cinerea* infection. Their results revealed the co-infection of biotrophic pathogen and necrotrophic pathogen can result in enhanced fungal resistance in plants, which is in line with the result in my study. They elucidated that the presence of flagellin or Pst helped plants to transduce fungal chitin signals more quickly, thereby enhanced plant defence against *B. cinerea (Gong et al., 2019)*. Flagellin has been shown to induce SA accumulation in plants and flagellin-triggered resistance to Pst is closely related to SA signalling (Tsuda, Glazebrook, et al., 2008; Tsuda, Sato, et al., 2008). In my study, the protective strains *P. vagans* C9-1 and *P. agglomerans* 299R which against Botrytis also strongly induced SA signalling response. These evidence suggest that SA signalling mechanisms might have an effect on resistance against necrotrophic pathogens. Moreover, plants might selectively regulate the sensitivity to different MAMPs such as flagellin and chitin to achieve optimized immune responses.

Overall, this study identified a number of bacterial strains that exhibit a promising feature as biocontrol agents against model pathogen Pst or *B. cinerea*. Beyond that, the protoplast assay confirmed that plants are able to recognize and differentially respond to non-pathogens. Indirect biocontrol mechanisms were dissected at species level, which furthers our understanding of the different modes of action of microbial biocontrol in the phyllosphere.

Table 5. 1 The combined results of chapter 2 and 4.

Strain	Protective ability against Pst	Pst population	SA response
Pedobacter sp. leaf 194	+	=	↓
Sphingomonas melonis FR1	+	=	=
Sphingomonas sp. leaf 34	+	=	NA
Sphingomonas sp. leaf 357	+	=	=
Plantibacter sp. leaf 1	+	=	=
Agreia sp. leaf 335	±	=	=
Microbacterium sp. leaf 347	±	=	=
Arthrobacter sp. leaf 145	±	=	=
Pseudomonas citronellolis P3B5	+	↓	↑
Sphingomonas phyllosphaerae FA2	+	=	↑
Sphingomonas sp. leaf 17	+	=	↑
Rathayibacter sp. leaf 296	±	=	↑
Pantoea vagans C9-1	±	=	↑
Pseudomonas syringae B728a	±	=	↑
Acidovorax sp. leaf 84	+	↓	=
Microbacterium sp. leaf 320	±	↓	=
Pseudomonas koreensis P19E3	±	↓	=
Pantoea agglomerans 299R	-	=	↑
Rhodococcus sp. leaf 225	-	=	↑
Erwinia amylovora CFBP1430S	-	↓	↓
Variovorax sp. leaf 220	-	↓	=
Methylobacterium radiotolerans 0-1	-	=	=
Bradyrhizobium sp. leaf 396	-	↓	NA
Methylorubrum sp. leaf 92	-	=	=
Axenic plants	-	=	=
Aeromicrobium sp. leaf 245	-	=	=
Williamsia sp. leaf 354	-	=	=
Methylobacterium sp. leaf 85	-	=	=

The protective ability against Pst is indicated by fully protective (+), intermediate protective (±), non-protective (-). Pst population suppression is indicated by "↓" as the reduction of mean Pst cell density compared to that of axenic plants is more than 10^2 cfu/g. "=" means there is no big difference in the average Pst cell density. The SA response is indicated by "↑" (more than 2 fold change compared to mock treatment or stronger induction than SA treatment), "=" (mild induction) and "↓" (weaker induction than mock treatment). Bacteria not tested in the protoplast assay were indicated by "NA".

5.2 Future work

5.2.1 Potential solutions for issues of the current experimental design

This study employed a gnotobiotic *in planta* assay in a 24-well plate system to determine the bacterial protective ability against pathogens by determining the disease symptoms of plant phenotypes. As mentioned in 2.4.2, it would be more accurate to quantify disease severity by using hyperspectral imaging technique (Lowe et al., 2017; Mahlein et al., 2012). In addition, some bacterial cell numbers could not exactly refer to their actual cell numbers on antibiotic agar since few strains were not fully resistant to that specific antibiotic. For example, *Pseudomonads* cell numbers would have 30 to 200 fold difference between Amp agar and R2A agar. This issue can be solved by employing a more sensitive and accurate qPCR method by using species-specific primers (Matsuda et al., 2007). In addition to the plant phenotypic screen, this study also employed a protoplast assay to determine the modulation of plant immune responses by bacteria. However, bacteria may behave differently in protoplast solution compared with natural leaves. For instance, Pst and *P. citronellolis* P3B5 exhibited a decreasing population in the presence of protoplasts. Since the protoplast buffer only contained glucose as carbohydrate source and solution osmolarity was designed for protoplast specifically, bacteria may lose their normal physiological function in a nutrient-deficient and hostile environment. Therefore, the protoplast buffer should be re-designed to be more suitable for both protoplasts and bacteria.

5.2.2 Future experiments

In this study, both, the population sizes of non-pathogens and Pst were determined in order to elucidate the potential microbe-microbe interactions between different bacteria. For *B. cinerea* infection, only plant phenotypes were determined due to the time constraints. These information were not enough to fully uncover the direct interactions between non-pathogens and pathogens. Furthermore, this study only determined SA signalling response, which limited information about other immune networks. Here, I propose improved technique for future studies.

5.2.2.1 Biochemical profiling

This study has shown that Pst population and fungal lesions were reduced by certain protective bacteria. This could be the result of direct antagonistic microbe-microbe

interactions or plant resistance activated by protective strains. Biochemical profiling is a useful method to analyse nutritional and metabolic capabilities such as carbon utilisation (Innerebner et al., 2011; Zengerer et al., 2018). In addition, this tool can determine the ability of certain bacteria to produce inhibitory metabolites (Chin-A-Woeng et al., 2003). Using biochemical profiling on protective bacteria can inform us whether they can produce antimicrobial products or share a nutrient-utilisation profile to directly antagonise pathogens. Genomic analysis would be a complementary way to simulate the ability of non-pathogens to produce certain antimicrobial products (Blin et al., 2019). Thus, this can further our understanding on direct microbe-microbe interactions on biocontrol activity.

5.2.2.2 Spatial distribution analysis

Due to the underlying heterogeneity of leaves, the abundance of bacterial populations at the whole leaf scale is insufficient to understand microbe-microbe interactions (Remus-Emsermann & Schlechter, 2018; Schlechter et al., 2019). A useful tool is spatial distribution analysis of non-pathogens and Pst at the single-cell resolution. This can tell us about how Pst are later colonised, adapted or antagonised on plant leaves in the presence of preemptive-colonised non-pathogens such as co-aggregation or segregation patterns (Schlechter et al., 2019). Also, for Botrytis infection, with the help of staining techniques of fungal hyphae, single-cell approaches can give a high-resolution picture of the inhibitory behavior of protective bacteria on fungal hyphae. This is also possible for the Botrytis infection experiments when combining bacterial single cell techniques with staining techniques.

5.2.2.3 Transcriptome study

Other than direct microbe-microbe interactions, plant hosts also play an important role in indirect microbe-microbe interactions (Legein et al., 2020). This study has only determined SA signalling response due to time limitations. In future experiments, pVSP1-eYFP-NLS or other transgenic lines can be employed to broaden our understanding of the role of the plant immune network in shaping microbial communities. Moreover, transcriptome study can be employed to determine the transcriptional response of plant hosts or microbes respectively. For instance, the presence of varying non-pathogenic bacteria might induce different plant transcriptional reprogramming before pathogen presence (Schlechter et al., 2019; Vogel et al., 2016). This cannot be observed through the leaf area. Transcriptomic studies will inform us about the different transcriptional responses before and after pathogen infection. However, it is worth mentioning that there could be a considerable heterogeneity in different plant cells as evidenced by my protoplast assay, which showed heterogeneous expression

of eYFP, in other words, different *PR1* promoter activities. Thus, transcriptome study at the single-cell resolution can provide a powerful approach to dissect cell heterogeneity.

5.2.2.4 Synthetic microbial community

This study used binary inoculations of non-pathogens and pathogens on plant leaves to investigate the drivers behind plant-protective effects. However, plants in nature live in association with a variety of microorganisms (Lindow & Brandl, 2003). It would be interesting to build a more complex synthetic microbial community to study whether these plant-protective effects are cumulative or synergistic. In the end, different modes of action of microbial biocontrol will be uncovered to better develop biocontrol strategies.

Supplementary Materials

Supplement Table 1 Fluorescent bacterial strains used in this study

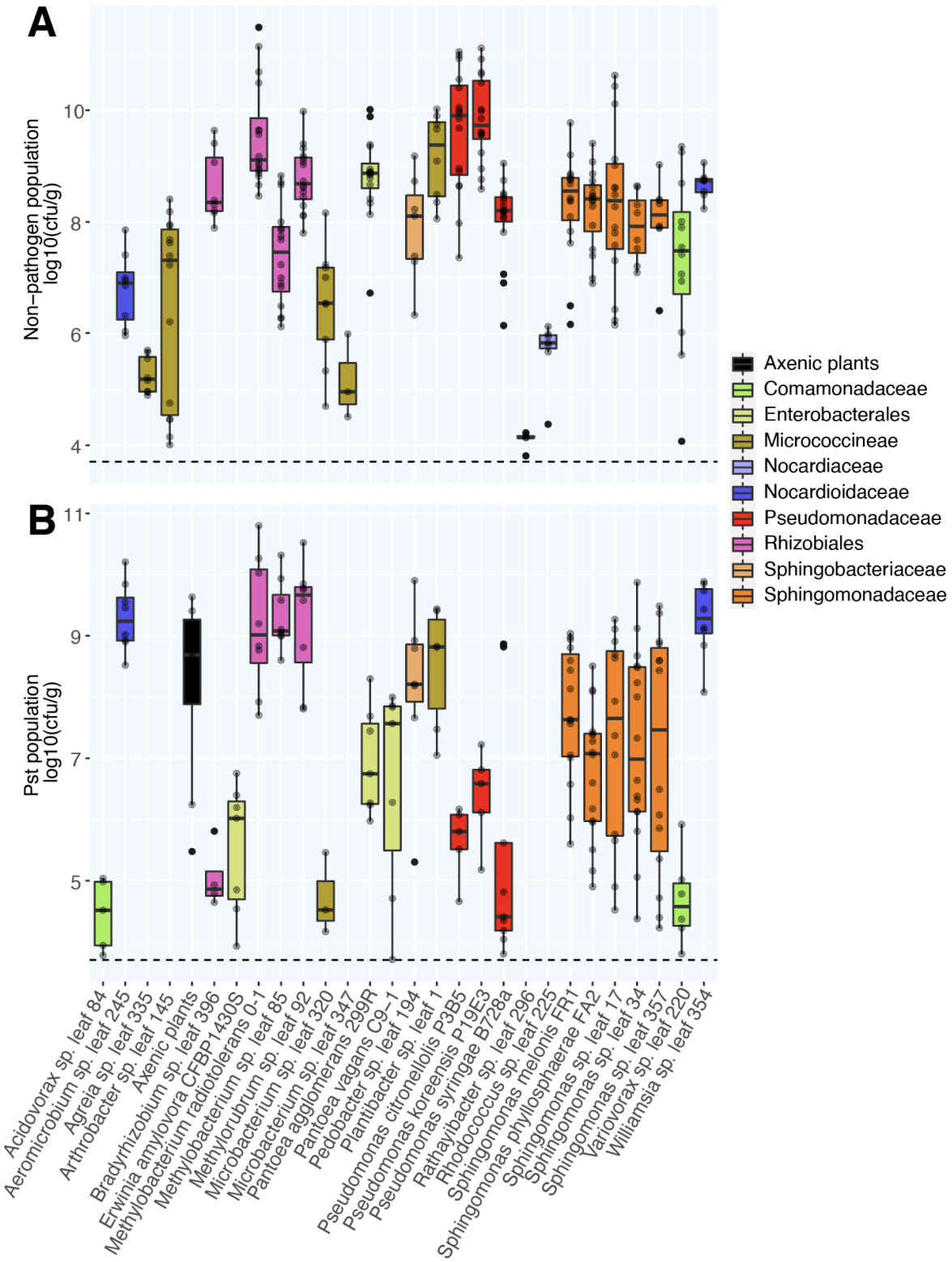
Bacterial strains	Fluorescence	Reference
Pseudomonas syringae DC3000 ::Tn7-145/2	mScarlet	(Miebach et al., 2020)
Sphingomonas phyllosphaerae::Tn5-145/1	mScarlet	(Schlechter et al., 2018)
Sphingomonas melonis::tn5-145/2	mScarlet	(Schlechter et al., 2018)
Sphingomonas sp. leaf 357::MRE-Himar-145/4	mScarlet	Unpublished, kind gift of Christian Stocks and Rudolf Schlechter
Methylobacterium sp. Leaf 85::tn5-145/1	mScarlet	(Schlechter et al., 2018)
Methylobacterium radiotolerans::tn5-145/1	mScarlet	(Schlechter et al., 2018)
Methylorubrum sp. eaf 92::tn5-145/1	mScarlet	(Schlechter et al., 2018)
Pseudomonas citronellolis::tn5-145	mScarlet	(Schlechter et al., 2018)
Pantoea agglomerans::Tn7-145	mScarlet	(Schlechter et al., 2018)
Acidovorax sp. Leaf 84::MRE-Himar-145/6	mScarlet	Unpublished, kind gift of Christian Stocks and Rudolf Schlechter

Supplement Table 2 The corresponding cell density when bacteria's OD_{600nm}= 0.5.

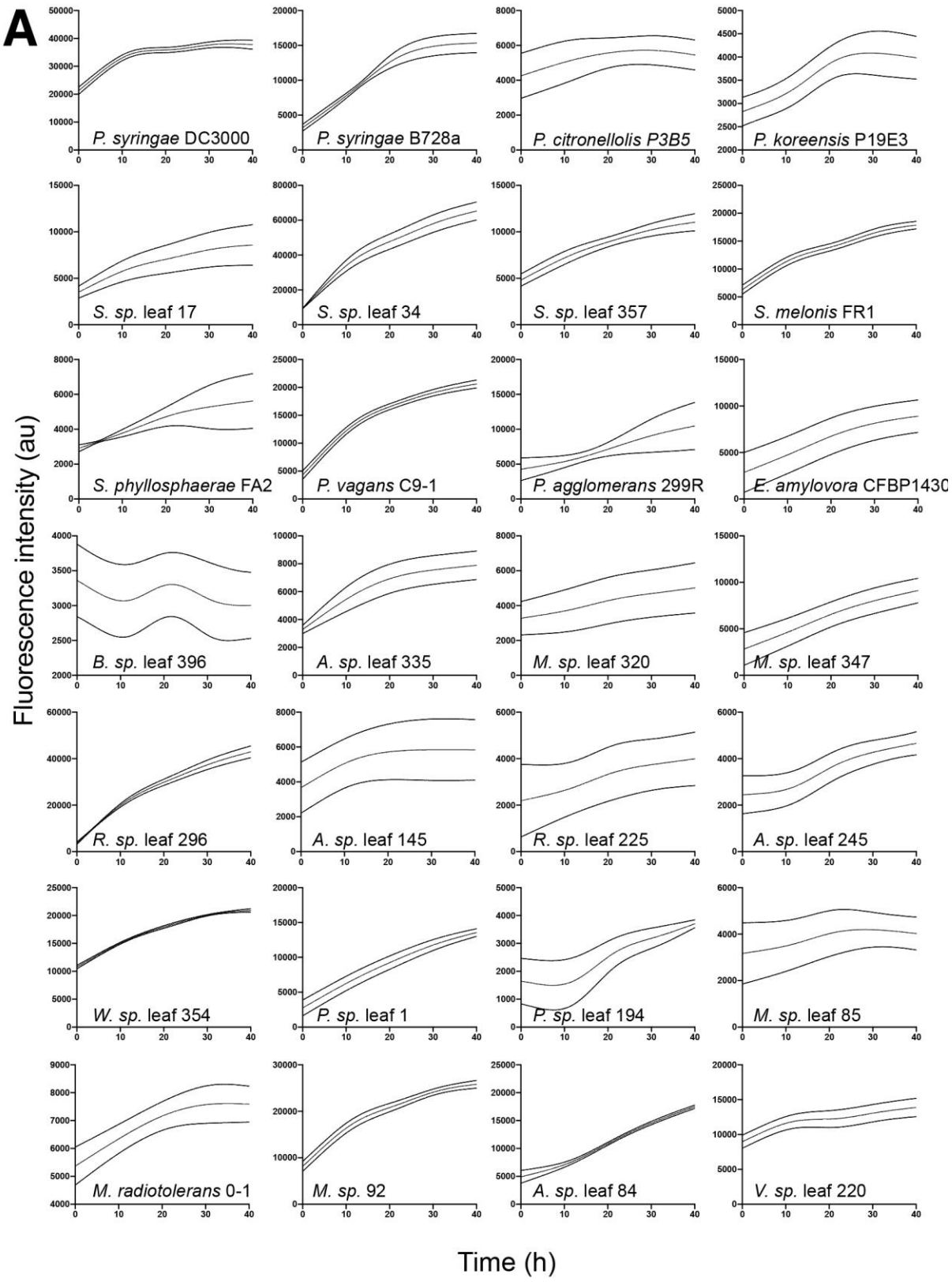
Strain	cfu/ml on R2A plate	log10(cfu/ml)
Pantoea agglomerans 299R	4.33E+08	8.64
Pantoea vagans C9-1	3.00E+08	8.48
Pantoea vagans PW	2.00E+08	8.30
*Pseudomonas syringa*e B728a	2.37E+08	8.37
Pseudomonas citronellolis P3B5	4.50E+08	8.65
Pseudomonas koreensis P19E3	4.50E+08	8.65
Erwinia amylovora CFBP14305	3.00E+08	8.48
Methylobacterium radiotolerans	5.83E+07	7.77
Methylobacterium sp. leaf 85	7.33E+06	6.87
Methylobacterium sp. leaf 92	3.33E+07	7.52
Sphingomonas melonis	8.67E+07	7.94
Sphingomonas phyllosphaerae	3.75E+08	8.57
Sphingomonas sp. leaf 17	4.00E+08	8.60
Sphingomonas sp. leaf 34	1.10E+07	7.04
Sphingomonas sp. leaf 357	1.48E+08	8.17
Bradyrhizobium sp. leaf 396	3.52E+08	8.55
Acidovorax sp. leaf 84	9.50E+07	7.98
Agreia sp. leaf 335	5.67E+08	8.75
Microbacterium sp. leaf 320	2.17E+09	9.34
Microbacterium sp. leaf 347	2.88E+08	8.46
Rathayibacter sp. leaf 296	1.43E+08	8.16
Arthrobacter sp. leaf 145	5.33E+08	8.73
Rhodococcus sp. leaf 225	5.00E+07	7.70
Aeromicrobium sp. leaf 245	7.83E+08	8.89
Williamsia sp. leaf 354	8.50E+07	7.93
Plantibacter sp. leaf 1	5.83E+08	8.77
Pedobacter sp. leaf 194	5.83E+08	8.77
Pseudomonas syringae DC3000	2.67E+07	7.43

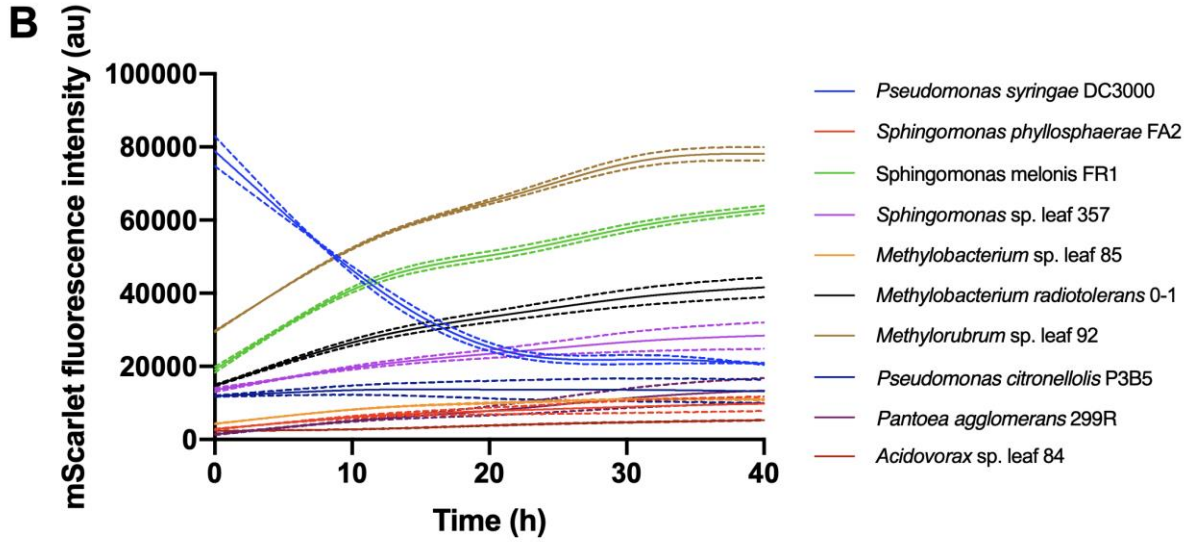
Supplement Table 3 The non-pathogen population development before and after the presence of Pst infection.

Pre-colonised strain	Before the presence of Pst	After the presence of Pst	Difference of mean
Pantoea vagans C9-1	9.05±0.35	<3.71	<-5.34 and >-9.05
Erwinia amylovora CFBP14305	8.81±0.58	*	*
Acidovorax sp. leaf 84	7.54±0.48	<3.71	<-3.83 and >-7.54
Microbacterium sp. leaf 347	8.33±0.47	5.15±0.76	-3.18
Agreia sp. leaf 335	8.10±0.25	5.26±0.33	-2.83
Rathayibacter sp. leaf 296	6.54±0.56	4.09±0.16	-2.45
Rhodococcus sp. leaf 225	7.86±0.12	5.68±0.59	-2.18
Arthrobacter sp. leaf 145	8.35±0.19	6.46±1.7	-1.89
Aeromicrobium sp. leaf 245	7.94±0.48	6.80±0.66	-1.14
Microbacterium sp. leaf 320	7.26±0.58	6.51±1.06	-0.75
Pseudomonas syringae B728a	8.57±0.44	8.03±0.74	-0.54
Williamsia sp. leaf 354	9.09±0.10	8.67±0.25	-0.42
Sphingomonas phyllosphaerae FA2	8.52±0.37	8.24±0.72	-0.27
Methylobacterium radiotolerans 0-1	9.80±0.17	9.54±0.93	-0.26
Pantoea agglomerans 299R	8.96±0.12	8.81±0.75	-0.15
Methylorubrum sp. leaf 92	8.92±0.47	8.78±0.57	-0.15
Sphingomonas melonis FR1	8.39±0.26	8.32±0.93	-0.08
Axenic plants	0	0	0.00
Sphingomonas sp. leaf 17	7.65±0.46	8.29±1.39	0.64
Sphingomonas sp. leaf 357	7.22±0.34	8.03±0.76	0.82
Plantibacter sp. leaf 1	8.28±0.37	9.17±0.78	0.88
Pseudomonas citronellolis P3B5	8.61±0.18	9.62	1.02
Pseudomonas koreensis P19E3	8.15±0.27	9.85	1.69
Variovorax sp. leaf 220	4.36±0.67	7.32	2.96
Pedobacter sp. leaf 194	4.93±0.55	7.89	2.97
Methylobacterium sp. leaf 85	<2.71	7.41±0.90	>4.7 and <7.41
Sphingomonas sp. leaf 34	<2.71	7.91±0.62	>5.20 and <7.91
Bradyrhizobium sp. leaf 396	<2.71	8.63±0.65	>5.92 and <8.63

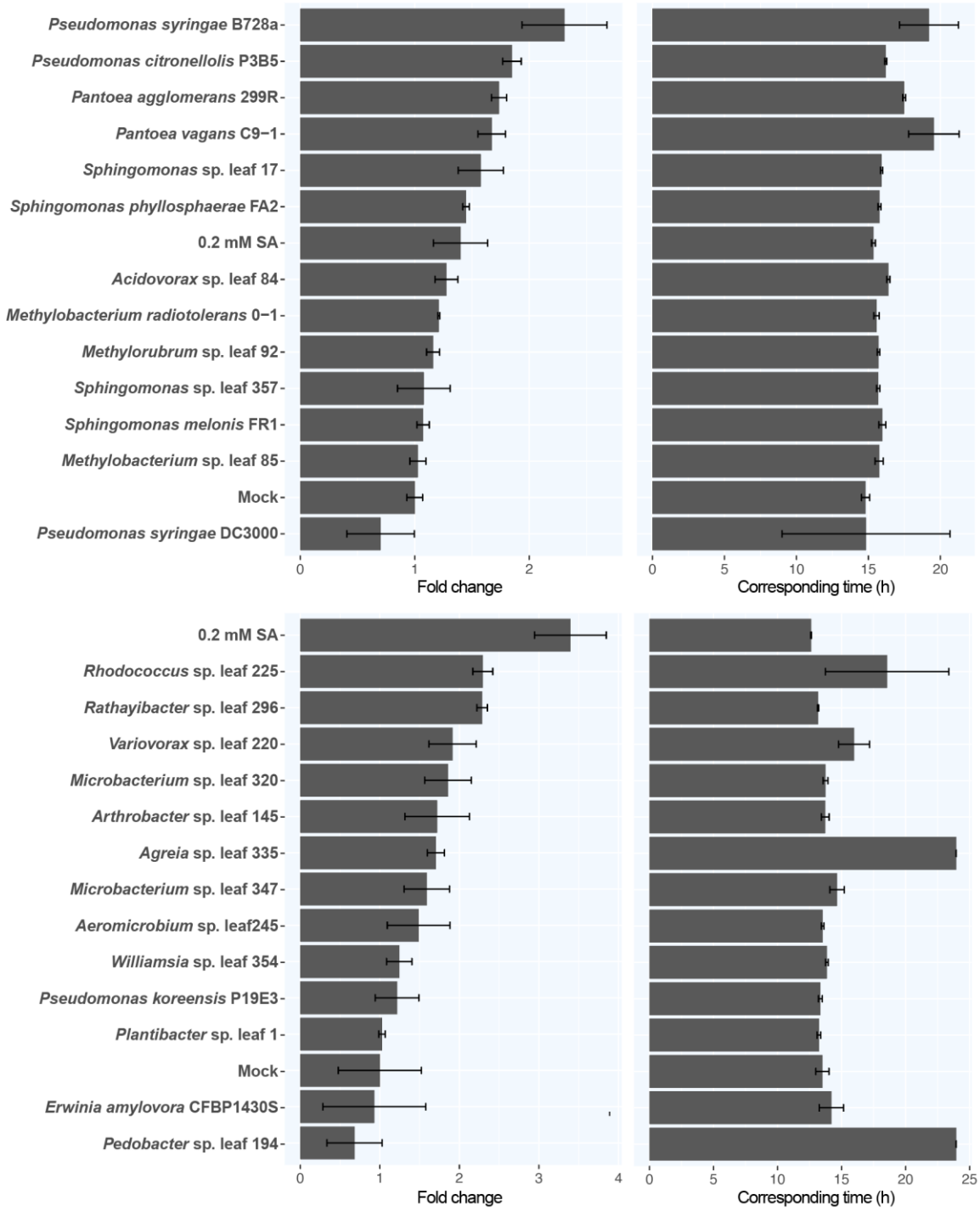


Supplement FIG 1. Non-pathogen population from plant phyllosphere (A) and the corresponding Pst population (B). Data were from two replicated experiments. Centerlines in box plots represent the 50th percentile and each box shows the interquartile range. Outlier dots represent values over 1.5 times the interquartile range beyond either end of the box. The bacteria population data only includes values above the counting threshold (3.71). Bacteria population from plants colonized by *Agreia* sp. leaf 335, *Microbacterium* sp. leaf 320, *Rathayibacter* sp. leaf 296, *Arthrobacter* sp. leaf 145, *Rhodococcus* sp. leaf 225, *Aeromicrobium* sp. leaf 245, *Williamsia* sp. leaf 354, *Plantibacter* sp. leaf 1, *Pedobacter* sp. leaf 194 and axenic plants were missing in the first experiment because high water temperature caused by long running sonic could kill the bacteria.

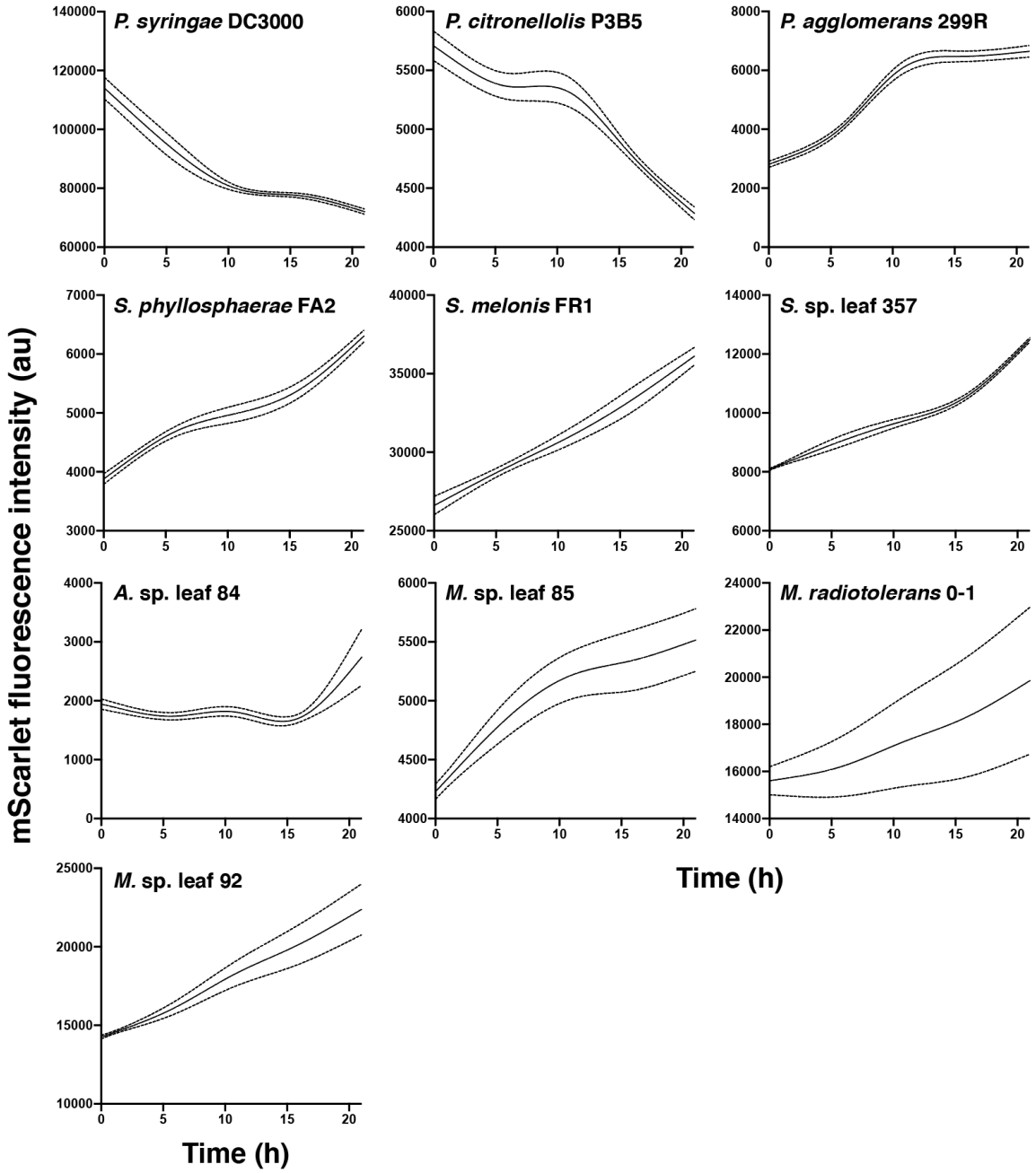

A
Fluorescence intensity (au)
Time (h)
P. syringae DC3000
P. syringae B728a
P. citronellolis P3B5
P. koreensis P19E3
S. sp. leaf 17
S. sp. leaf 34
S. sp. leaf 357
S. melonis FR1
S. phyllosphaerae FA2
P. vagans C9-1
P. agglomerans 299R
E. amylovora CFBP1430
B. sp. leaf 396
A. sp. leaf 335
M. sp. leaf 320
M. sp. leaf 347
R. sp. leaf 296
A. sp. leaf 145
R. sp. leaf 225
A. sp. leaf 245
W. sp. leaf 354
P. sp. leaf 1
P. sp. leaf 194
M. sp. leaf 85
M. radiotolerans 0-1
M. sp. 92
A. sp. leaf 84
V. sp. leaf 220



Supplement FIG 2. Bacterial autofluorescence detected by the yellow fluorescence channel and their corresponding mScarlet fluorescence. Bacteria were cultured in W5 buffer for 40 hours to test bacterial autofluorescence and their behaviour in the absence of protoplasts. Each bacterial treatment had three biological replicates. Data was removed background signals and smoothed. The standard deviation of each time point was between dashed lines. (A) Bacterial autofluorescence (B) Bacterial behavior of mScarlet tagged strains.



Supplement FIG 3. Fold change of peak yellow fluorescence intensities of Fig 4.1A compared to mock treatment and their corresponding time. Error bar indicated the standard deviation of three biological replicates. Results of two independent experiments were separated. Strain names were sorted by peak yellow fluorescence intensity value.



Supplement FIG 4. Bacterial behavior of mScarlet tagged bacteria in the presence of transgenic protoplasts. Each bacterial treatment had three biological replicates. Data was removed from background signals (W5 buffer) and smoothed. The standard deviation of each time point was between dashed lines.

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